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VOLUME 36 SEPTEMBER 1958 NUMBER 5

EFFECTS OF TEMPERATURE TREATMENT AND OF A NATIVE INHIBITOR ON SEED DORMANCY AND OF COTYLEDON REMOVAL ON EPICOTYL GROWTH IN VIBURNUM TRILOBUM MARSH[1]

ROBERT HUGH KNOWLES AND SAUL ZALIK

Abstract

Dormancy was encountered in both seeds and seedlings of *Viburnum trilobum* Marsh. When seeds were exposed to germinative conditions at 20° C., dormancy was expressed by slow growth of the radicle and hypocotyl in some seeds, and by lack of germination in others. This type of dormancy was found to be associated with the presence of a water-soluble inhibitor, as well as with a need for an appropriate temperature treatment. Epicotyl dormancy, or failure of shoot growth following seedling emergence, was overcome by removal of the cotyledons.

Introduction

Plant breeders frequently encounter difficulty in obtaining satisfactory germination of the seed of high-bush cranberry (*Viburnum trilobum* Marsh.), and its hybrids. If allowed to germinate under natural conditions without special treatment, this species seldom produces seedlings earlier than the second season after planting. In addition, even though the root system develops after germination, the epicotyl of the seedling remains dormant for an indefinite period. Under laboratory conditions, the seed will germinate slowly at 20° C., but has been reported to require exposure to a temperature of 5° C. following germination to induce shoot production (4).

Since the germination of many dormant seeds is usually favored by temperatures between 2° and 5° C. (7), the fact that *V. trilobum* germinates at 20° C. suggests a special type of dormancy. Although failure of shoot growth following germination is not unique to *Viburnum* (1, 2, 3, 6), complete development of the seedling is complicated in that continued exposure of germinated seeds to 20° C. in some way prevents epicotyl development.

The studies reported here deal with the influence of temperature and seed coats on germination, and the effect of cotyledons on epicotyl dormancy.

Materials and Methods

The seeds used in these studies were harvested in the fall of 1956 from plants growing on the University of Alberta campus. In order to avoid complications which might result from soaking or steeping the fruit prior to seed removal, seeds were separated from the fruit by crushing, leaving the endocarps intact. Then, the seeds were rinsed in running water and dried at room temperature.

[1]Manuscript received April 11, 1958.
Contribution from the Department of Plant Science, University of Alberta, Edmonton, Alberta.

[The previous number of Can. J. Botany (**36**, 439–559) was issued July 17, 1958.]

Thirty-eight hundred seeds were used in the germination studies; 1900 of these were decorticated by completely removing the endocarp from the seed prior to planting. The seeds were planted in vermiculite in shallow, waxed-paper containers of 135 ml. capacity. Twenty-five seeds were placed in each of the containers, which were then transferred to controlled temperature chambers. The germination medium was moistened every 7 days, and seeds were examined periodically to observe germination. To facilitate these examinations, the contents of each container were placed in a sieve and held under a stream of tap water to wash away the vermiculite. The seeds were then replanted in fresh vermiculite.

Water extracts of dried ground endocarps were prepared by shaking 3-g. samples of material in 100 ml. of water for 48 hours. To test the effects of these extracts on the growth of roots, uniform samples of *Viburnum* seedlings and of wheat (*Triticum vulgare* var. Canus) were placed on filter paper in Petri plates to which aliquots of the extract had been added. Root measurements were made on the wheat seedlings after 24 hours, and on the *Viburnum* seedlings after 10 days.

An experiment was designed to gain information on the temperature and light requirements of the epicotyl for afterripening. Two uniform lots of seedlings (from decorticated seeds), with radicles 1½ in. in length, were transplanted to pots containing vermiculite. These were maintained in the dark, one lot at 5° C., the other at 20° C. Samples of these were brought into constant light at room temperature after 2 and 4 weeks' dark treatment.

To study the effect of the cotyledons upon afterripening of the epicotyl, samples which had been in the dark for 2 weeks at 5° C., and at 20° C., were transferred to continuous light. After 2 weeks, the cotyledons were removed from half of each lot. Fluorescent tubes were used to supply a constant light intensity of 300 ft-c. The number of seedlings showing epicotyl growth was recorded periodically.

Experimental Results

Germination Studies

The effects of several temperature treatments on germination of whole and of decorticated seeds were studied over a period of 156 days. The treatments employed, and the results obtained, are shown in Table I.

During the first 110 days, the maximum germination attained was 47% for decorticated seeds held at 20° C. constant temperature, and 30% for intact seeds maintained at this temperature. Germination did not exceed 2% for any of the other treatments during this period. However, when seeds which had been exposed to alternating temperatures for 110 days were transferred to constant 20° C. for several weeks, not only was germination obtained, but the percentage germination in all cases was significantly better than that resulting from exposure to the most favorable constant temperature.

Indications of an Inhibitor

Steeping of the seed while in the germination medium might be harmful if a water-soluble inhibitor were present in either the endocarp or testa. To determine if an inhibitor was present 400 decorticated seeds were rinsed eight times prior to being placed in the germination medium. The germination of these was compared with 400-seed samples of whole and of unrinsed decorticated seeds.

TABLE I

GERMINATION OF *V. trilobum* AFTER 110 DAYS' TREATMENT FOLLOWED BY EXPOSURE FOR 46 DAYS TO 20° C.

Temperature pre-treatment in ° C. for 110 days	Germination percentages after 46 days at constant 20° C.	
	Intact seeds	Decorticated seeds
Constant		
2° C.	4	20
5° C.	4	20
20° C.	36	63
Alternating		
2°–20°, daily	75	83
2°–20°, 7 days	59	97
2°–20°, 14 days	47	96
5°–20°, daily	91	80
5°–20°, 7 days	86	89
5°–20°, 14 days	60	82
Mean	51	70

NOTE: L.S.D. at 5%, seed type × temperature, 22.

The data in Table II show that endocarp removal and rinsing of the testa reduced the time to 50% germination, and markedly increased the percentage germination over the controls. However, even this treatment did not result in more than 59% germination at the termination of the experiment.

TABLE II

EFFECT OF ENDOCARP REMOVAL AND OF RINSING ON SUBSEQUENT GERMINATION OF THE SEED OF *V. trilobum*

Treatment	Percentage germination after given number of days		
	50	66	80
Check intact seed	1	12	22
Endocarp removed; testa not rinsed	18	29	42
Endocarp removed; testa rinsed	52	59	59

A partial explanation for the favorable effect on germination of rinsing decorticated seeds is evident from the results obtained when wheat and *Viburnum* seedlings were grown in endocarp extracts. The 48-hour extract inhibited root growth in seedlings of both plants very markedly (Table III).

TABLE III

EFFECT OF ENDOCARP EXTRACTS ON GROWTH OF WHEAT AND *Viburnum* SEEDLINGS

Treatment	Root length, mm.	
	Wheat	*Viburnum*
Water check	13.0	23.5
Extract	2.8	10.8

NOTE: Concentration of original extract, 30 mg. per ml.

Epicotyl Dormancy and Afterripening

In preliminary experiments dealing with seedling development it was found that epicotyl dormancy could be overcome completely by exposure of *Viburnum* seedlings to 5° C. for 3 months, but the data in Table IV show that afterripening occurred to a considerable extent in seedlings exposed to both 5° C. and 20° C.

TABLE IV

EFFECT OF PRETREATMENT OF *Viburnum* SEEDLINGS ON SHOOT PRODUCTION WHEN TRANSFERRED TO LIGHT AT ROOM TEMPERATURE

Pretreatment in dark	Percentage of seedlings with shoots after given number of days in light		
	30	45	55
2 weeks at 5° C.	0	37	50
4 weeks at 5° C.	7	40	67
2 weeks at 20° C.	3	27	33
4 weeks at 20° C.	10	47	50
None	0	0	10

TABLE V

EFFECT OF COTYLEDONS ON GROWTH OF *Viburnum* EPICOTYLS

Dark treatment preceding cotyledon removal	Percentage of seedlings with epicotyls after 24 days in light at room temperature	
	Seedlings with cotyledons intact	Cotyledons removed after seedlings in light for 14 days
2 weeks at 5° C.	2	100
2 weeks at 20° C.	0	100

PLATE I

FIG. 1. Effect of cotyledon removal on epicotyl growth after 24 days' exposure of *Viburnum* seedlings to light at room temperature. *a*, Seedlings with intact cotyledons and no epicotyl development. *b*, Epicotyl development on seedlings with excised cotyledons.

The most striking result in the experiments on epicotyl dormancy was produced by removing the cotyledons (Fig. 1). The immediate epicotyl growth in 100% of the seedlings from which the cotyledons had been removed (Table V) is very significant. Even after 2 months' exposure to light, epicotyl development had not occurred in more than 50% of the seedlings with intact cotyledons, which had received the 2 weeks' dark exposure.

Discussion

In considering the data of Table I relating to endocarp removal and temperature treatment, it should be observed that interpretation is complicated at this stage (156 days) by the effect of five rinsings employed in earlier examinations, which might have removed any soluble inhibitor if one were present. The data of Tables II and III clearly indicate that there are one or more such soluble substances in the seed which inhibit both germination and root elongation.

There was a general increase of 19% germination with removal of the endocarp (Table I). This difference is statistically significant. Giersbach (5), in her studies on several species of *Viburnum*, noted no such beneficial effect from endocarp removal, but *V. trilobum* was not among the species she studied.

Although there appears to be ample evidence that a water-soluble germination inhibitor is present, it should be observed that removal of the endocarp, followed by rinsing (Table II), did not result in as high germination as when endocarp removal was accompanied by alternating temperature treatments (Table I). It seems, therefore, that a combination of these, namely, the removal or the dilution of an inhibitor, and the use of alternating temperatures prior to exposure to constant 20° C., was required for optimal germination.

Considering the data of Tables IV and V it seems that epicotyl afterripening in *V. trilobum* will occur in the light following exposure in the dark to either 5° C. or 20° C. These results are not in agreement with the findings of Giersbach (5), who noted that a temperature of 5° C. was required for the afterripening of dormant epicotyls in all the northern species of *Viburnum* which she studied. Clearly, in these studies, epicotyl growth of *V. trilobum* was governed mainly by the cotyledons. Although the mode of this control is not known, possible explanations might be: that a competitive relationship exists between the cotyledons and the epicotyl for food reserves; that the cotyledons inhibit the epicotyl in a manner similar to that by which apical buds inhibit the growth of laterals; or that a combination of these two may be operative.

The failure of *V. trilobum* seed to respond promptly when exposed to the germinative conditions employed in this study was thus found to be associated with the presence of a water-soluble inhibiting substance in the endocarp and with a requirement for an inductive temperature treatment prior to exposure at constant 20° C. On the other hand, failure of epicotyl development following germination was influenced more by the presence of cotyledons than by postgerminative temperature treatments.

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DISCOVERY OF FOSSIL CERATOPHYLLUM DEMERSUM L. IN NORTHWEST TERRITORIES, CANADA[1]

J. Terasmae and B. G. Craig

Abstract

Fossil *Ceratophyllum demersum* L. was discovered on sites east of Great Slave Lake, Northwest Territories, Canada. This distribution is both north and east of its presently known range. The palynological evidence supports the conclusion that the climate was warmer than the present when the silt, in which the fossil plant remains were found, was deposited. A radiocarbon age of 5400 ± 230 years (L-428) has been obtained for the sample.

In 1955 during geological reconnaissance of the area east of Great Slave Lake, Northwest Territories, a pingo was examined by B. G. Craig in the floodplain, or perhaps an abandoned channel of the Thelon River, at latitude 64° 19′ north and longitude 102° 41′ west.

A closer examination revealed that the pingo was composed of stratified silt and the upper three to four feet contained abundant plant remains along some of the bedding planes. A sample of this material was collected by Craig and later submitted for radiocarbon analysis by him.

A palynological and palaeobotanical analysis of the sample was made by Terasmae and the evidence from this study suggested some rather interesting implications of the results, relating to the vegetation and its habitat at the time of deposition.

It was during the palaeobotanical examination of the sample that leaves of *Ceratophyllum demersum* L. were discovered and later identified under the microscope because only the characteristically forked tips of the leaves were found intact. Several comparisons were made with herbarium specimens of aquatic plants and the authors wish to acknowledge the kind assistance and suggestions of W.K.W. Baldwin and H. J. Scoggan of the National Museum of Canada during this phase of the identification. Details of the fossil leaves were photographed and are shown in Fig. 1. These details were perfectly matched on a specimen of *Ceratophyllum demersum* L. in the National Herbarium of Canada (No. 13223, collected by E. C. Smith *et al.* at Antigonish, Nova Scotia). The identification is supported by the description given by Gleason (2), who also points out that it is our only genus of submerged plants with leaves whorled and palmately dissected. The present study shows that the position and the size of the unicellular spines is significant, as well as the cell structure of the leaves and the characteristic forking of the leaves.

When the ecological, and perhaps especially the climatological, significance of the fossil *Ceratophyllum demersum* L. in the Thelon River Valley was considered in combination with the palynological evidence, presented in Table I, three intriguing conclusions were reached.

[1]Manuscript received April 28, 1958.
Contribution from the Geological Survey of Canada, Ottawa, Canada. Published with the permission of the Director, Geological Survey of Canada.

First, this evidence suggests that the past vegetation, at the time when the silts were deposited, was different from the present. A forest cover, perhaps as isolated groves on ecologically suitable sites, must have existed to account for the abundance of tree pollen.

Second, the presence of *Ceratophyllum* suggests a habitat, such as a shallow pond or an isolated river channel on a broad floodplain, in which silting occurred probably periodically.

TABLE I

POLLEN ASSEMBLAGE ISOLATED FROM THE SILT SAMPLE

Species	Number of pollen grains counted
Pinus banksiana	13
Picea mariana	29
Picea glauca	48
Alnus sp.	7
Betula cf. *papyrifera*	6
Betula cf. *glandulosa*	25
Salix sp.	3
Artemisia sp.	1
Cyperaceae	3
Equisetum sp.	Present

Third, the climatic conditions at the time were definitely not glacial, in fact the climate was warmer than the present because there is no forest growth, except for a few isolated trees, in the area now (3) and the known northern distribution limit of *Ceratophyllum demersum* L. is well south of the Great Slave Lake area (1, 5). According to Scoggan (5) the distribution of *Ceratophyllum demersum* L. extends from British Columbia and northern Alberta to central Manitoba and east and south to Nova Scotia and Central America. The northernmost specimen was collected by H. M. Raup (No. 2345) on July 28, 1928, in the Murdock Creek district (the Wood Buffalo Park) at latitude 59° 14′ north and longitude 111° 34′ west. This specimen is at the National Museum of Canada (Herbarium No. 55104).

Consequently the evidence seems to indicate that the silt was deposited during a postglacial climatic episode warmer than the present, the postglacial thermal maximum. This episode has been dated by the radiocarbon method to have occurred at 6000 to 7000 years B.P. in eastern North America and Europe. Allowing for a lag in the time parallelism of the pollen zones in a south–north direction a probable age of 5000 to 6000 years B.P. may be estimated for the analyzed silt sample.

The formation of the pingo is necessarily a later event and may coincide with the marked cooling of climate following the postglacial thermal maximum.

It was most encouraging when the obtained radiocarbon date (L-428) for the silt sample showed an age of 5400 ± 230 years B.P. It is also interesting to note in this connection that Porsild (4) examined pingos in MacMillan Pass on the Canol Road (Mile 225 E.) and noted that "the pingos were rather small and immature, the largest being 150 by 50 feet and about 10 feet high.

PLATE I

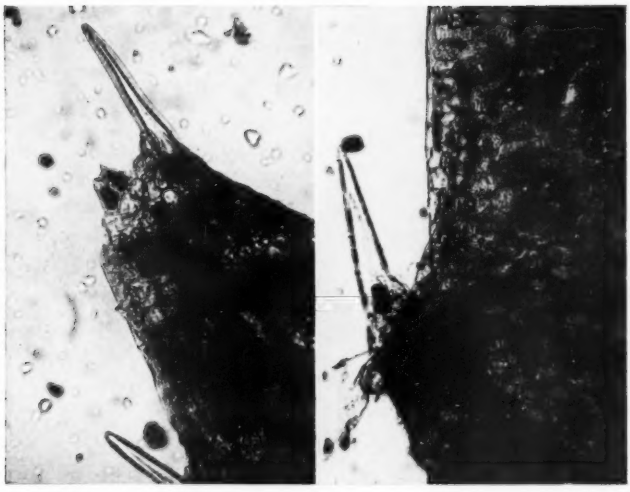

FIG. 1. Photomicrographs of leaves of fossil *Ceratophyllum demersum* L. Length of spines, 150–170μ.

Pits dug into the summit of the pingos showed a fine-grained, homogeneous, black peat. In the peat were numerous well-preserved rhizomes of *Menyanthes trifoliata* and other lacustrine plants, showing that the peat was laid down in shallow water. *Menyanthes* does not now grow in the swamp, nor elsewhere in MacMillan Pass, a fact that may indicate a deterioration of climate. Permafrost was found at a depth of 23 inches".

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THE INHERITANCE IN WHEAT OF A BLUE ENDOSPERM COLOR DERIVED FROM AGROPYRON ELONGATUM[1]

D. R. KNOTT

Abstract

The gene(s) for blue endosperm was found to be carried on an *Agropyron* chromosome which is not homologous to a wheat chromosome. The gene(s) is partially dominant and has a distinct dosage effect.

A wheat having blue endosperm and designated "Blue A" was developed by Dr. B. C. Jenkins (formerly at the University of Saskatchewan, now at the University of Manitoba) from the backcross, [*Triticum vulgare* Vill. variety Rescue ($2n = 42$) × *Agropyron elongatum* (Host) Beauv. ($2n = 72$)] × Rescue. The chromosome number of Blue A was not determined but it is reasonably stable and wheat-like in morphology. Since the blue color is an endosperm character, it was of interest as a possible genetic marker in studies to determine the frequency of natural crossing in wheat. For this reason cytogenetic studies were undertaken.

Morrison and Rajhathy (1) studied natural hybrids between Thatcher and Blue A but did not obtain clear-cut results. They concluded that segregation for color was actually a segregation for either a fragment or a whole chromosome.

Materials and Methods

When this study was begun, homozygous blue F_4 lines from a cross between Thatcher and Blue A were available. During the development of the lines, selection for deep-blue color had been practiced but no record was kept of the segregation for color.

Two F_4 lines were crossed reciprocally with Thatcher and the resulting F_1 plants were backcrossed to Thatcher. A number of plants from each generation were examined cytologically and the color of their seeds recorded.

Results and Conclusions

The results of the cytological studies are given in Table I.

The two parental blue lines had deep blue-black seeds and 21 pairs of chromosomes. The hybrid seeds from the crosses with Thatcher were intermediate in color but the reciprocal crosses differed in the intensity of the blue. When plants from either of the blue lines were used as the maternal parents, the hybrid seeds were clearly blue although not as dark as those of the blue parent. However, when Thatcher was the maternal parent the hybrid seeds were only faintly tinged with blue. Since the endosperm is triploid and derives two of its chromosome complements from the maternal

[1]Manuscript received March 14, 1958.
Contribution from the Department of Field Husbandry, University of Saskatchewan, Saskatoon, Saskatchewan.

parent, the blue-black seeds of the parent lines would have three doses of the gene or genes for blue color and the seeds from the reciprocal crosses would have two doses and one dose respectively. It appears, therefore, that three doses of the gene or genes for color are necessary to produce the blue-black seeds of the parents. Two doses condition a medium blue and one dose results in a faint tinge of blue.

TABLE I

RESULTS OF CYTOLOGICAL STUDIES ON THE PARENT BLUE LINES AND ON PLANTS FROM THE CROSS AND BACKCROSS TO THATCHER

Generation	Number of plants	Chromosome configuration	Endosperm color of seed*
Thatcher–Blue A F_1	11	21_{II}	Blue
(Thatcher–Blue A × Thatcher) F_1 (or reciprocal cross)	17	$20_{II} + 2_I$ or $18_{II} + 1_{IV} + 2_I$	Seg. blue†
	1	$19_{II} + 3_I$	Seg. blue
(Thatcher–Blue A × Thatcher) × (Thatcher) F_1	12	$20_{II} + 1_I$	Red
	4	21_{II}	Red
	4	$20_{II} + 2_I$	Seg. blue
	1	$21_{II} + 1_I$	Seg. blue

*Since this is an endosperm character, the seeds on an F_1 plant will segregate for color.
†Seg. = segregating.

All but one of the F_1 plants from the cross with Thatcher had a configuration of either $20_{II} + 2_I$ or $18_{II} + 1_{IV} + 2_I$. The presence of two univalents indicates that the blue parents carried one chromosome which was not homologous to a Thatcher chromosome. This chromosome must have come from the *Agropyron* parent so that the constitution of the blue lines was 20 pairs of wheat chromosomes plus 1 pair of *Agropyron* chromosomes. The exceptional F_1 plant had one fewer chromosomes than its sibs and had three univalents rather than two. Since the absence of the *Agropyron* chromosome would have left only one univalent it is evident that one wheat chromosome had been lost. Only the normal F_1 plants were used in the backcrosses to Thatcher.

In the backcross to Thatcher the F_1 plants were used as the maternal parents. If the letter "W" is used to designate wheat chromosomes and "A" to designate the *Agropyron* chromosome, then the F_1 plants had the constitution, $20_{II}W + 1_IW + 1_IA$. Sears (2, 3) has shown in wheat that a univalent frequently either lags or misdivides during meiosis and is lost, with the result that the chromosome is present in about one fourth rather than the expected one half of the gametes. If the two univalents in the F_1 plants behave in this way, then each would be present in one quarter of the gametes. Thus both chromosomes would be present in 1/16 of the gametes, each would be present singly in 3/16, and both would be absent in 9/16 (a 9:3:3:1 ratio).

From the backcross four types of plants would be expected, also in the ratio 9:3:3:1 (see Table II). As is shown in the lower part of Table I, the four types appeared with a frequency of 12:4:4:1, which is a good fit to the expected ratio. It is apparent that all four types of eggs functioned. The five plants which carried an *Agropyron* chromosome produced seeds which segregated for blue color, while the sixteen plants which lacked the *Agropyron* chromosome had only red seeds. It is concluded, therefore, that the gene (or genes) for blue color is on the *Agropyron* chromosome.

TABLE II

TYPES OF GAMETES PRODUCED BY THE F_1 PLANTS AND CHROMOSOME CONSTITUTION OF THE PLANTS FROM THE BACKCROSS TO THATCHER

Type of gamete	20W	21W	20W + 1A	21W + 1A
Frequency	9	3	3	1
Constitution of backcross plants	$20_{II}W + 1_{I}W$	$21_{II}W$	$20_{II}W + 1_{I}W + 1_{I}A$	$21_{II}W + 1_{I}A$

The frequency of red and blue seeds was recorded for a number of plants of known chromosomal constitution. If it is assumed that plants having a configuration of $20_{II}W + 1_{I}W + 1_{I}A$ produce four types of gametes in a ratio of 9:3:3:1 as suggested above, that all types are equally viable, and that no type has a competitive advantage, then the expected F_2 ratio is 7 seeds carrying the *Agropyron* chromosome to 9 lacking it. The recorded segregation for such plants was 304 blue:338 red seeds. This is a fair fit to a 7:9 ratio ($P = .05$–$.10$), although the frequency of blue seeds tends to be high. That the segregation approximates a 7:9 is surprising since it is highly improbable that there is no competition among pollen of the four different chromosome types. In monosomic wheat plants it has been shown by Sears (2, 3) that the 21-chromosome pollen functions almost to the exclusion of the 20-chromosome pollen. The same relationship almost certainly would be true here and should have resulted in a large excess of blue seeds. The absence of a large surplus of blue seeds indicates that any failure of the 20-chromosome pollen to function is counterbalanced by reduced functioning of the pollen carrying an *Agropyron* chromosome.

Only one of the plants studied cytologically had a configuration of $21_{II}W + 1_{I}A$ but another plant had the same proportion of blue seeds and was evidently of this constitution. On these plants the F_2 segregation approximated 1 blue:3 red seeds with the actual numbers being 13 blue:41 red. Assuming that the univalent behaved normally, the parent plants should have produced two types of gametes, 21W and 21W + 1A, in the ratio of 3:1. If both types functioned equally well an F_2 ratio of 7 blue:9 red seeds would be expected. The number of seeds is small but it is evident that there is a significant shortage of blue seeds. Since no sterility was observed in the

plants it appears that all of the eggs function but that pollen carrying the *Agropyron* chromosome is at a disadvantage in competition with normal pollen.

The author has also studied stem rust resistance derived from *Agropyron elongatum* and found it to be conditioned by a gene or genes on a single *Agropyron* chromosome. F_2 ratios, similar to those for blue color, were obtained. A number of blue-seeded plants were tested and found to be rust susceptible. It is probable, therefore, that the genes for blue color and rust resistance are on different *Agropyron* chromosomes.

The observations made in this study indicate that seeds carrying a single *Agropyron* chromosome have only a faint tinge of blue. The faintness of the color makes classification difficult except in very good seed samples. It is doubtful, therefore, that the blue endosperm character is of value in experiments to measure the frequency of natural crossing in wheat.

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THE PHYSIOLOGY OF STOMATA

II. THE APPARENT ABSENCE OF CHLOROPHYLL, PHOTOSYNTHESIS, AND A NORMAL RESPONSE TO LIGHT IN THE STOMATAL CELLS OF AN ALBINO BARLEY[1]

MICHAEL SHAW[2]

Abstract

The chlorophyll content of the pure albino plants produced by self-fertilizing a strain of variegated barley is about 1.2 μg. per gram fresh weight. The stomatal guard cells of the albino plants are normal in structure, but do not contain detectable amounts of chlorophyll. Microradioautography of epidermal strips previously exposed to radioactive carbon dioxide in light and darkness did not reveal the localization of radioactivity which would be expected in the guard cells if they were capable of photosynthesis. Moreover, the "albino" stomata do not open normally in response to light or in a carbon-dioxide-free atmosphere. These observations support the view that normal, light-sensitive stomatal cells contain functional chloroplasts and that stomatal opening is promoted by the photosynthetic removal of free carbon dioxide in the mesophyll and in the guard cells.

Introduction

In Part I it was shown that chlorophyllous stomatal guard cells, whether or not they normally contain starch, apparently photosynthesize at a low rate (9). Stomatal opening is promoted by small reductions in the carbon dioxide content of the surrounding air (4, 5). Thus one way in which light may promote stomatal opening is through the photosynthetic lowering of the concentration of free carbon dioxide at centers in the guard cells. The importance of "mesophyll" photosynthesis in this connection is strongly suggested by the fact that the apparently chlorophyllous stomata on the "albino" portions of variegated leaves, such as variegated *Pelargonium*, open less widely than the stomata over green mesophyll (Table III in (5)). This view is further supported by the observation that, in carbon-dioxide-free air, the stomata over the "albino" mesophyll of *Pelargonium* open almost as widely in the light and just as widely in the dark as those over green mesophyll (Table III in (5)).

Six years ago I found it impossible, by illumination or other treatment, to induce stomatal opening in the albino offspring produced by a strain of variegated barley (1, 2). This paper describes some experimental observations on the stomatal physiology of these albino plants carried out mainly between 1952 and 1955.

Material and Methods

The mutation "variegated" arose spontaneously from a six-awned, rough-bearded barley (1). When the variegated plants were self-fertilized, about 90% of the offspring were pure albino and Arnason *et al.* (1) concluded from

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this and other genetic data that a mutant gene induced an irreversible plastid change which was inherited maternally. Seed, produced by selfing "variegated", was supplied by Dr. T. J. Arnason and was sown in pots or flats in the greenhouse. Observations were made on the first and second leaves of variegated and albino seedlings 6 to 12 days after planting.

Stomata were examined on living epidermal strips as described in (8) and in phenolic IKI (Heath's reagent: (see 3)) after fixation by Lloyd's method. The Molisch test (reduction of 1% $AgNO_3$ brought to pH 4.0 with acetic acid) was carried out and the fluorescence of chlorophyll demonstrated as described in (8). The activity of phosphorylase was demonstrated cytochemically by incubation with glucose-1-phosphate (7).

The plastid pigments were extracted from the leaves and the extracts examined in a Beckman spectrophotometer (model DU, 1 cm. cells) essentially as described in (9). The techniques employed in exposing leaves and isolated epidermis to radioactive carbon dioxide and in subsequent microradioautography have also been described in (9).

Experimental Results

1. Chlorophyll Content and Photosynthesis in Albino Leaves

It was first demonstrated by fluorescence microscopy (8) that the small mesophyll plastids of albino leaves did, in fact, contain traces of chlorophyll. The results of chlorophyll analyses are presented in Table I. The absorption spectra of ether solutions of the pigments from albino and normal green leaves are shown in Fig. 1. The presence of both chlorophylls *a* and *b* in the "albino" extract was demonstrated by a paper chromatographic method.

TABLE I

CHLOROPHYLL ESTIMATIONS (1953)

Variety	Total chl.*	Chl. *a**	Chl. *b**	*a*/*b*
Albino	1.21	0.73	0.46	1.59
Montcalm (green)	770	580	196	2.96

*μg./g. fresh weight of leaves.
NOTE: The carotenoid content of the albino was about 0.75% that of Montcalm leaves.

Albino leaves were exposed to radioactive carbon dioxide in light and darkness. From autoradiographs and counts of the dried leaf material, it was estimated that the rate of carbon dioxide fixation in the light was 2- to 4-fold that in the dark. It was therefore concluded that the albino leaves photosynthesize at a very low rate. It is possible that the rate of photosynthesis was limited by the virtual absence of stomatal opening (see below).

2. Observations on Stomatal Cells

The stomatal guard cells of normal green barley leaves contain pale-green chloroplasts which reduce silver nitrate, give a positive test for starch and for phosphorylase, and have been shown to fix carbon dioxide (9). The guard

cells of albino seedlings appeared to be normal in structure (Fig. 2), and possessed small plastids which gave a positive test for starch and for phosphorylase and which reduced silver nitrate (8). Reduction of silver nitrate renders plastids easily visible (8); it does not necessarily indicate the presence of chlorophyll. No green color could be detected in the "albino" guard cells; nor was it possible to detect the characteristic red fluorescence of chlorophyll under any conditions (8). These are, however, negative results and it follows that, if there is chlorophyll in the "albino" guard cells, it is present only in the very minutest amounts.

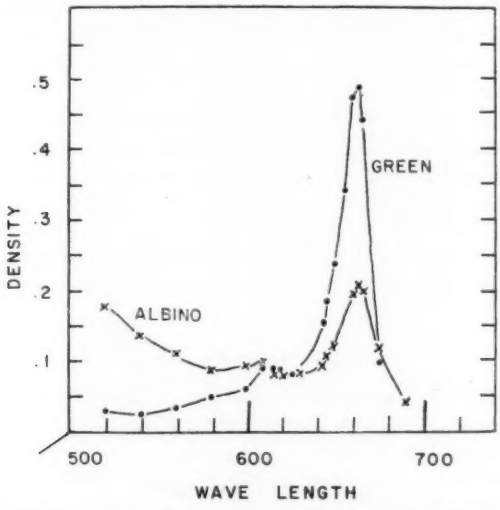

FIG. 1. Absorption spectra of ether extracts of normal green (0.0075 g. fresh weight/ml.) and albino (2.5 g. fresh weight/ml.) barley leaves. Ordinate: optical density. Abscissa: wave length in millimicrons.

Intact albino leaves with their bases in water and epidermal strips floating on water (with the cuticle side both up and down) were exposed to radioactive carbon dioxide (approximately 0.03% v/v; total activity 15–20 μc.) in light and darkness for periods of 3 or 4 hours. Microradioautography yielded no evidence for the localization of radioactivity in the guard cells. These are, again, negative results, but the experiment was repeated several times and the techniques employed have given clear evidence that a light-enhanced fixation of carbon dioxide occurs in normal chlorophyllous guard cells (see, in particular, Fig. 11 in (9)). Absence of guard-cell photosynthesis is also suggested by the observation that, after the food reserves of the seed are exhausted, starch completely disappears from the guard cells of albino leaves.

3. Stomatal Aperture and Movement

Repeated microscopic examinaton at high magnification of intact leaves, living epidermal strips, and alcohol-fixed strips, taken from plants growing in

the greenhouse as well as from plants kept under artificial light and in darkness, revealed no evidence of normal stomatal opening at any time of day. Nearly all the stomata appeared tightly closed. Occasional stomata (probably $<1\%$) on epidermal strips were slightly open. This may be attributed to the effects of stripping. Judging from the size of the stomatal apparatus, and observations on normal green barley leaves, the maximal width of the stomatal pore to be expected under conditions favoring opening is from 6 to 10 μ.

Several attempts were made to induce stomatal opening by exposing both detached leaves and intact seedlings to a stream of carbon-dioxide-free air in both light and darkness. No opening occurred under conditions which caused normal opening in green leaves. It therefore appears certain that the stomata of this albino barley do not show a *normal* response to light or to a lowered partial pressure of carbon dioxide.

Supporting this conclusion are several observations. Young albino leaves were generally more turgid and wilted less rapidly after excision than normal green leaves. Virgin (10) has stated that "nonchlorophyllous" barley leaves produce "guttation" droplets under conditions, conducive to transpiration, which prevent guttation from normal green leaves. I am able to confirm this point. Further, Virgin (10) has shown by measurements with the "corona hygrometer" that "nonchlorophyllous" leaves have a low rate of transpiration.

Discussion

Virgin (10) reported the complete absence of chlorophyll from the leaves of a radiation-induced "albino" mutant of barley. However, he used a relatively weak ether extract of the leaves (0.073 g. fresh wt./ml.) in measuring the absorption spectrum of the pigments present (see his Fig. 5). Use of a larger amount of leaf tissue (see Fig. 1) might have led to the detection of amounts of chlorophyll of the order of magnitude reported in Table I.

There is approximately the same amount of chlorophyll in the leaves of albino barley (Table I) as in the epidermis of tulip (1.46 μg./g. fresh weight. See Table I in (9)). Arnason and Walker (2) counted 40–50 mutated plastids per mesophyll cell of the albino barley and the bulk of the chlorophyll in the albino leaves is certainly present in the mesophyll. If the fresh weight of the epidermis is taken as 10% of the fresh weight of the leaf and if it is assumed further that 10% of the chlorophyll present in the leaves is in the epidermis, then the guard cells of the albino could, theoretically, contain as much chlorophyll as those of tulip (see Table I in (9)). If this were true, the plastids in the albino guard cells should be a striking green color. They appear colorless. The only possible conclusion seems to be that the mutated plastids in "albino" guard cells contain at the most only a small fraction of the chlorophyll found in "green" guard cells.

To summarize, it has been shown (i) that the leaves of the pure albino segregating from variegated barley (1, 2) contain traces of chlorophyll and photosynthesize at a very low rate; (ii) that the "albino" guard cells virtually lack

PLATE I

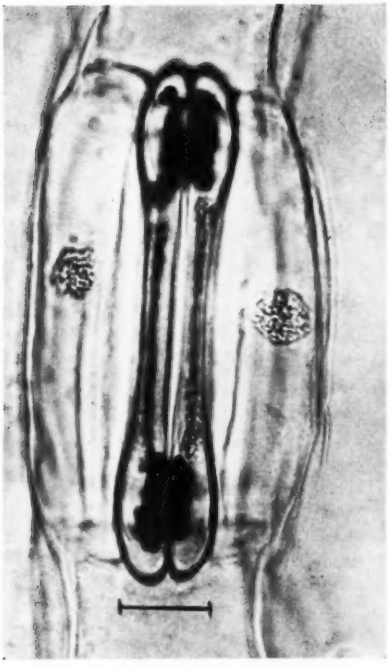

FIG. 2. Stomatal apparatus of albino barley. Alcohol-fixed strips mounted in phenolic IKI. Horizontal line = 10 μ.

chlorophyll and do not fix carbon dioxide in the light at a rate which is detectable by radioautography under the conditions employed; and (iii) that the "albino" stomata do not open normally in response to illumination or in the absence of carbon dioxide.

If these findings are valid, they support the conclusion of Scarth and Shaw (6) that the effect of light on stomatal opening "depends on photosynthesis and, mainly at least, on reduction of CO_2 concentration in the leaf"; and the conclusion of Shaw (8) that "chloroplasts may occur in the guard-cells of all light-sensitive stomata, whether or not these normally contain starch." They also support Virgin's (10) more recent conclusion that normal light-induced stomatal movement depends on photosynthesis in the leaf.

Finally, it must be pointed out that possibly only the use of the highly sensitive porometer technique could determine whether in fact the stomata of albino barley show absolutely no response to light. However, any actual response must be of a minor order of magnitude and, if substantiated, would scarcely alter the main conclusions developed here.

Acknowledgments

I am grateful to Miss Ann Oaks for the chlorophyll analyses which are incorporated in a thesis presented to the Graduate Faculty for the M.A. degree, to Dr. G. A. Maclachlan for carrying out some of the tracer experiments, and to the National Research Council for a grant.

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PHOTOSYNTHESIS AND METABOLISM IN MARINE ALGAE

III. DISTRIBUTION OF PHOTOSYNTHETIC CARBON FROM $C^{14}O_2$ IN FUCUS VESICULOSUS[1]

R. G. S. Bidwell,[2] J. S. Craigie,[3] and G. Krotkov[3]

Abstract

F. vesiculosus plants were permitted to carry on photosynthesis in sea water in the presence of either $Na_2C^{14}O_3$ or $C^{14}O_2$, and the radioactive products of photosynthesis were examined by chromatography. The main compound in the ethanol-soluble fraction was mannitol, which contained up to 95% of the soluble activity. Citric acid and glutamine also accumulated considerable activity. Alginic acid was the main insoluble product of photosynthesis and appeared to be an active metabolite. Combined fucose also became radioactive, and was shown to be present in two forms. It was released from one by mild acid hydrolysis, but from the other only after much stronger hydrolysis. Neither the addition of nutrient salts to the sea water, nor increase in the amounts of CO_2 or carbonate supplied, caused much variation in the relative distribution of C^{14} among the photosynthetic products.

Introduction

It is well known that many compounds found in seaweeds differ from those found in land plants, and it has been shown that the soluble products of photosynthesis are characteristic of the various groups of algae (3). The brown algae are characterized by accumulation of the sugar alcohol, mannitol, by lack of soluble sugars such as sucrose, glucose, or fructose, and by their polysaccharides, the more important being laminarin, fucoidin, and alginic acid. Various authors have speculated on the roles of these compounds in the metabolism of brown algae, but conclusions have been drawn on circumstantial evidence only (5, 6, 11, 13). To determine directly which of these compounds are important products of photosynthesis, experiments with $C^{14}O_2$ were carried out on the brown rockweed, *Fucus vesiculosus*, and the ethanol-soluble and -insoluble products of photosynthesis have been examined.

Materials and Experimental Techniques

Plants

Material was collected at low tide from Point Pleasant, Halifax, N.S., and used at once. The sea temperature at the time of collection varied from 3° C. in early spring to 13° C. in summer. Experimental material consisted of sterile fronds cut into 2 cm. lengths, in randomly selected samples of 2.0 g. fresh weight (10 to 15 pieces).

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[3]Biology Department, Queen's University, Kingston, Ontario.

Procedure

Prepared plant samples were placed in sea water in special glass chambers kept at 16° C. and illuminated by incandescent lights at 1600 ft-c. C^{14} was supplied either as $Na_2C^{14}O_2$ solution or as $C^{14}O_2$ in the gas phase bubbled through the water by circulation in a closed system. Uptake of C^{14} was determined either by counting barium precipitates of aliquots of sea water when $Na_2C^{14}O_3$ was supplied, or by automatic recording monitors when $C^{14}O_2$ gas was supplied. At the end of each experiment the plants were rinsed quickly with distilled water and extracted with 50% ethanol. The extracts were chromatographed, radioautographed, and the radioactive spots were eluted, plated, and counted in a methane-flow counter. Corrections were made for self absorption where necessary. All activities were corrected to absolute values. The photosynthesis apparatus, experimental procedure, and the analytical techniques used have been described in detail in Part II of this series (3).

Experiments and Results

Alcohol-Soluble Products of Photosynthesis

In preliminary experiments with $Na_2C^{14}O_3$, it was found that the main alcohol-soluble product of photosynthesis in *F. vesiculosus* was mannitol (3). It was thought desirable to test the possible effects of variation of the external conditions on the production of mannitol. The first experiment was run to determine the effect of added nutrient salts on photosynthesis. Four plant samples were run for 24 hours in the manner described above. They were supplied with $Na_2C^{14}O_3$, with various amounts of added potassium nitrate and sodium phosphate. The experimental conditions and results of analyses are shown in Table I.

TABLE I

EXPT. 1: EFFECT OF SALTS ON PHOTOSYNTHESIS OF *Fucus vesiculosus**

	Salts supplied			
	0	0.2×normal†	Normal	5×normal
Activity uptake, μc.	7.3	7.5	8.1	7.2
Soluble, μc.	5.5	6.4	6.5	5.4
Residue, μc.	1.8	1.1	1.6	1.8
CO_2 uptake, mg.	7.3	7.5	8.1	7.2
Activity in soluble compounds, as percentage of soluble activity				
Mannitol	76	74	68	71
Citric acid	11.4	10.2	12.4	11.5
Aspartic acid	0.8	0.6	0.8	0.3
Asparagine	1.7	2.4	2.3	2.6
Glutamic acid	3.8	3.4	3.8	3.8
Glutamine	6.5	9.0	12.4	11.3

*Sample weight, 2.0 g. in 100 ml. sea water at 16° C., illumination 1600 ft-c. 24 mg. $Na_2C^{14}O_3$=10.0 μc. were supplied to each sample. Duration, 24 hours.
†Normal salts: 10 mg. KNO_3+2 mg. $Na_2HPO_4.12H_2O$ per 100 ml. of sea water.

It may be seen from Table I that the added nutrient salts had little effect on either the total uptake of C^{14} or its distribution in the plants. There was, however, a slight decrease of activity in mannitol and small increases in glutamine and asparagine. The large amounts of radioactivity in citric acid and glutamine are noteworthy.

A second experiment was conducted to determine the effect of $Na_2C^{14}O_3$ concentration on photosynthesis. Four samples of plants were placed in sea water containing various amounts of $Na_2C^{14}O_3$ and illuminated for 24 hours. The conditions of the experiment and results of analyses are shown in Table II.

TABLE II

EXPT. 2: EFFECT OF $Na_2C^{14}O_3$ CONCENTRATION ON PHOTOSYNTHESIS OF *Fucus vesiculosus**

$Na_2C^{14}O_3$ supplied, mg.	6	12	24	48
Activity supplied, μc.	5	10	20	40
Activity uptake, μc.	4.5	8.5	17.2	25.2
Soluble, μc.	2.4	4.7	8.9	13.8
Residue, μc.	2.1	3.8	8.3	11.4
CO_2 uptake, mg.	2.3	4.3	8.6	12.6
Activity in soluble compounds, as percentage of soluble activity				
Mannitol	78	75	72	74
Citric acid	11.1	11.7	13.5	11.6
Aspartic acid	—	0.5	0.4	0.6
Asparagine	—	—	0.6	0.7
Glutamic acid	4.0	3.7	3.8	3.4
Glutamine	6.3	7.3	7.3	7.3
Unknowns	—	2.3	2.1	2.1

*Sample weight, 2.0 g. in 100 ml. sea water at 16° C., illumination 1600 ft-c. Duration, 24 hours.

About 90% of the C^{14} supplied to plants was assimilated by the three samples with the smaller amounts of Na_2CO_3, but the fourth sample took in only 63%. In this sample the pH rose to 8.8 after about 6 hours and photosynthesis stopped. It may be seen that though the total amounts of CO_2 taken in by various samples varied from 2.3 to 12.6 mg., the distribution of activity between soluble and insoluble fractions and among soluble compounds in various samples was virtually unaffected.

It was concluded from the above experiment that supplying C^{14} as sodium carbonate was impractical because of the tendency of the pH to rise. Therefore, in the third experiment varying quantities of $C^{14}O_2$ in air were supplied to plants by bubbling the gas mixture through the medium in a closed circulating system for 6 hours. This practice did not affect the pH of the medium sufficiently to disturb the plants, even at a CO_2 concentration in the air of 10%. The amounts of $C^{14}O_2$ supplied in this experiment and the results of analyses are shown in Table III.

It may be seen that between 60 and 80% of the activity offered was assimilated by various samples, and that the distribution of activity between the soluble and insoluble fractions was about the same in all samples. However, the proportion of activity in mannitol was considerably greater than in

previous experiments, and this tended to increase with increasing CO_2 concentration. The amounts of radioactivity in citric acid and glutamine were much lower than before, and they decreased with increasing CO_2 concentration.

From Table II it is seen that the sample which was supplied with the largest amount of carbonate took in 12.6 mg. of CO_2. In this experiment the plants ceased to assimilate CO_2 after 6 hours because of the high pH. From Table III it may be seen that the sample provided with the highest CO_2 concentration took in 16 mg. of CO_2 in 6 hours of photosynthesis. Since in this last sample the CO_2 was 80% exhausted at the end of the experiment, it is possible that its rate might have been even higher if more CO_2 had been present.

TABLE III

EXPT. 3: EFFECT OF $C^{14}O_2$ CONCENTRATION ON PHOTOSYNTHESIS OF *Fucus vesiculosus**

CO_2 supplied, mg.	2.5	5	10	20
% CO_2 in air	1.25	2.5	5	10
Activity supplied, μc.	5	10	20	40
Activity uptake, μc.	3	7	14	32
Soluble, μc.	2.2	5.0	10.4	22.0
Residue, μc.	1	2	4	10
CO_2 uptake, mg.	1.5	3.5	7.0	16
Activity in soluble compounds, as percentage of soluble activity				
Mannitol	89	88	91	95
Citric acid	4.7	3.3	2.8	1.0
Aspartic acid	—	—	—	0.2
Glutamic acid	3.4	3.7	2.7	1.5
Glutamine	3.2	3.8	2.2	1.4
Valine	—	—	0.4	0.5
Unknowns	—	1.3	1.9	1.0

*Sample weight, 2.0 g. in 100 ml. sea water at 16° C., illumination 1600 ft-c. Duration, 6 hours. CO_2 supplied in air, by bubbling.

Experiment 4 was carried out to determine the maximum rate possible for CO_2 assimilation and the time during which this rate could be sustained. A sample of plants was placed in sea water in the CO_2 circulating apparatus and supplied with $C^{14}O_2$ at an initial concentration of 10% CO_2 in air. The apparatus was flushed out and recharged when the concentration of CO_2 became low. The $C^{14}O_2$ concentration in the circulating air was recorded and is shown in Fig. 1. The rate of CO_2 uptake by the plants was calculated from this curve, using the assumption that the specific activity of $C^{14}O_2$ in the apparatus was not diluted appreciably during the experiment by respired $C^{12}O_2$. Experimental conditions of this experiment and the uptake of CO_2 are shown in Fig. 1.

It may be seen from Fig. 1 that the rate of CO_2 assimilation was high initially, but soon steadied at the rate of about 3–4 mg. CO_2 per gram fresh weight per hour. The same behavior was observed after recharging of the CO_2 in the circulating air. At the last recharging, at 29.5 hours, the plants became suddenly inactive and so were removed and extracted. It is apparent

that over the range of 1% to 8% of CO_2 in the gas phase and for the period of up to 28 hours, the assimilation rate of *F. vesiculosus* is independent of CO_2 concentration in the atmosphere.

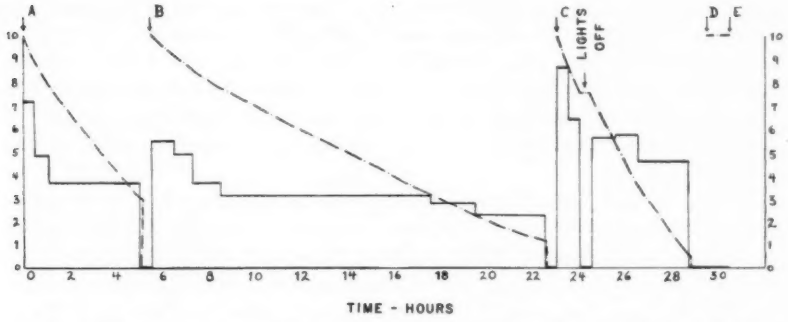

FIG. 1. Expt. 4: Relationship between the concentration of CO_2 and the rate of CO_2 uptake by *Fucus vesiculosus*. The apparatus was charged with 30 mg. of CO_2 at times A, C, and D. Its volume was doubled and it was charged with 60 mg. of CO_2 at time B. The plants were killed at E. Sample weight, 2.0 g. in 100 ml. sea water at 16° C., illumination 1600 ft-c. — · — · — · — CO_2 concentration in circulating air, as per cent v/v. ———— Rate of CO_2 uptake, as mg. CO_2 per g. fresh wt. per hour.

Experiment 4 was run in May, 1957. In Experiment 5, run under similar conditions in July, 1957, the results were essentially the same except that the steady rate of CO_2 uptake was about 1.4 mg. CO_2 per gram fresh weight per hour. The results of analysis of soluble fractions from experiments 4 and 5 are shown in Table IV. In both experiments the mannitol content was high. Both citric acid and glutamine were low, and considerable activity appeared in the several unknown spots. Some of these unknown spots gave glucose or mannitol on hydrolysis.

TABLE IV

EXPTS. 4 AND 5: DISTRIBUTION OF ACTIVITY IN *Fucus vesiculosus* AFTER 30 HOURS OF PHOTOSYNTHESIS IN $C^{14}O_2$*

	Expt. 4	Expt. 5
Activity taken, μc.	85.7	90.0
Soluble, μc.	58.5	60.0
Insoluble, μc.	29.0	30.0
CO_2 assimilated, mg.	206	108
Activity in soluble compounds, as percentage of soluble activity		
Mannitol	78	82
Citric acid	2.4	0.4
Aspartic acid	0.4	0.3
Glutamic acid	0.6	0.4
Glutamine	0.2	0
Alanine	0.1	0
Unknowns	19	17

*Sample weight, 2.0 g. in 100 ml. sea water at 16° C., illumination 1600 ft-c.

Alcohol-Insoluble Products of Photosynthesis

It was of interest to determine whether radioactive carbon entered insoluble carbohydrates during photosynthesis. The ethanol-soluble residues of experiments 1–4 were extracted and "soluble alginate" and fucoidin were prepared from each in the manner of Black *et al.* (10). Some radioactivity was associated with these fractions, but only traces of identifiable compounds with negligible activity appeared on their hydrolysis. The residues remaining after this extraction were then hydrolyzed. Chromatograms of the hydrolyzates contained small amounts of amino acids but no carbohydrates and almost no activity. The "acid humin" formed during this hydrolysis was strongly radioactive.

In a search for a more effective extraction procedure, the residue from experiment 5 was divided into two equal parts. In procedure A, applied to one half, the residue was twice extracted overnight with dilute HCl, pH 2.2, at 70° C., then washed with water and the washings added to the dilute HCl extract. The residue was then twice extracted overnight with 3% Na_2CO_3 at 50° C. The HCl extract was made to 30%, then 60%, and finally to 90% with ethanol. Precipitates which separated were redissolved in very dilute ammonia water, plated, and counted. The 90% ethanol-soluble material was also counted. The Na_2CO_3 extract was treated similarly, except that ethanol concentrations of 50% and 80% were used for precipitation. The residue after carbonate extraction was hydrolyzed in 3 *N* HCl at 105° C. for 18 hours, and the hydrolyzate was counted and chromatographed. The small amount of insoluble humin formed during this hydrolysis was burned in O_2 in a furnace at 1242° C., and the resulting CO_2 was precipitated as $BaCO_3$, plated, and counted. All the precipitates as well as the ethanol-soluble material were hydrolyzed in *N* HCl for 3 hours at 100° C., and the resulting hydrolyzates were counted and chromatographed.

In procedure B, applied to the other half of the residue from experiment 5, the residue was hydrolyzed first with *N* HCl at 100° C. for 3 hours. The residue from this hydrolysis was then hydrolyzed with 3 *N* HCl at 100° for 6 hours. The insoluble humin formed during the last hydrolysis was burned in O_2 at 1242° C. as above. The hydrolyzates were chromatographed and their activities determined. The amounts of radioactivity in the extracts and hydrolyzates obtained by these two methods are shown in Table V.

In procedure A, a large amount of activity was found in the precipitates which formed in both the dilute HCl and the carbonate extracts on the addition of ethanol to 30 or 50% respectively. These precipitates were insoluble in water or dilute acid, and resisted hydrolysis in 0.5 *N* HCl at 100° C. They were readily soluble in 0.5 *N* NaOH or NH_4OH, from which they were precipitated either on the addition of 10% $CaCl_2$ solution or on the addition of ethanol to 50%. The calcium precipitates were hydrolyzed overnight at room temperature in 80% H_2SO_4, and yielded a compound which was identified by chromatography as mannuronic acid. It is concluded that the material in both these precipitates was alginic acid.

TABLE V

EXPT. 5: RECOVERY OF ACTIVITY FROM ALCOHOL-INSOLUBLE RESIDUE OF *Fucus vesiculosus* AFTER 30 HOURS' PHOTOSYNTHESIS IN $C^{14}O_2$*

Fraction	Activity, μc.	Composition
Procedure A		
Dilute HCl extract, pH 2.2, 70° C.		
30% ethanol precipitate	7.14	Alginic acid
60% ethanol precipitate	0.29	
90% ethanol precipitate	7.60	Some fucose, xylose, unknowns released on hydrolysis
90% ethanol soluble	2.98	
3% Na_2CO_3 extract, 50° C.		
50% ethanol precipitate	3.01	Alginic acid
80% ethanol precipitate	0.17	
80% ethanol soluble	0.69	
Hydrolysis of residue in 3 *N* HCl, 105° C., 18 hr.	2.72	Amino acids of protein
Combustion of insoluble humin	0.67	
Total	25.3	
Not accounted	4.7	
Procedure B		
Hydrolysis in *N* HCl, 100° C., 3 hr.	15.32	Glucose, galactose, fucose, xylose, unknowns
Hydrolysis in 3 *N* HCl, 100° C., 6 hr.	4.14	Glucose, galactose, fucose, unknowns
Combustion of insoluble humin	2.52	
Total	22.0	
Not accounted	8.0	

*Experimental conditions are given in Table IV. Total activity in residue, 30.0 μc. Recovery figures for each procedure have been doubled to give values for the whole residue.

TABLE VI

EXPT. 5: DISTRIBUTION OF ACTIVITY AMONG INSOLUBLE COMPOUNDS IN *Fucus vesiculosus* AFTER 30 HOURS' PHOTOSYNTHESIS IN $C^{14}O_2$*

Compound	Activity, μc.	Determined by procedure:
Alginic acid	10	A
Xylose	0.6	B
Fucose	4.6	B
Glucose + galactose	2.4	B
Insoluble humin	0.6	A
Unknown 5	0.5	A
Unknown 53	1.8	B
Amino acids	1.6	A
Total	22.1	
Not accounted	7.9	

*Experimental conditions are given in Table IV. Total activity in residue, 30.0 μc.

The next most radioactive material was the 90% ethanol precipitate from the dilute HCl extract. Some radioactive fucose and xylose were released from this on hydrolysis in *N* HCl at 100° C. for 3 hours, but most of the radioactivity was associated with insoluble humin which formed during hydrolysis. Some radioactive fucose, xylose, and several unknowns were released on hydrolysis of the 90% ethanol-soluble fraction of the dilute HCl extract, but again much of the activity was lost on hydrolysis (not accounted for).

The insoluble humin remaining after the last hydrolysis of procedure A corresponds approximately with the material which has been considered to be cellulose in brown algae (7).

A summary of the distribution of activity in various compounds isolated chromatographically from hydrolyzates following procedure B, together with the protein amino acids, alginic acids, and insoluble humin obtained following procedure A, is shown in Table VI. Included in this table are unknown 5, a fast-running spot giving no spray reaction (2,3), and unknown 53. The latter gives the characteristic orange color of hydroxymethylfurfural (HMF) with aniline-phthalate, but unlike HMF runs quite slowly on chromatograms (R_{Alanine}: 0.48 in butanol – acetic acid – water and 0.38 in phenol). None of the unknowns obtained following procedure B, which might have arisen from degradation of compounds listed in procedure A, are included, to avoid the possibility of counting any of the radioactivity twice. Even so, almost three quarters of the radioactivity in the insoluble residue has been located in these given compounds. Alginic acid contained most of the activity, fucose was next, then glucose and galactose. The last two compounds did not separate completely one from another on chromatograms and hence are listed together, but glucose was always greatly in excess of galactose.

Discussion

Three major points of interest arise from these experiments. The first is the production of mannitol in large amounts as the major soluble product of photosynthesis. The second point is the constancy of the relative distribution of photosynthetic carbon among various fractions and compounds under different external conditions. The third point is that alginic acid, not laminarin or fucoidin, appears to be the most rapidly accumulated insoluble product of photosynthesis.

The rapid appearance of such a large proportion of photosynthetic carbon in mannitol suggests a possible method for the biosynthesis of radioactive mannitol on a laboratory scale. Such a synthesis has been carried out, and the details are published elsewhere (4).

The lack of effect of added salts on rates and products of photosynthesis is interesting. Black and Dewar (9) found a correlation between seasonal variations in constituents of algae with the concentration of nutrient salts in the sea, and concluded that the rate of photosynthesis depends on the amounts of nitrate and phosphate in the sea. Their conclusion was not substantiated in the present experiment.

The formation of larger amounts of radioactive alginic acid than of any other insoluble carbohydrate is of great interest. It has been shown by microscopic study that alginic acid is primarily located in the middle lamella of cells of *Laminaria*, like the pectic acid of higher plants (1). However, in similar experiments with leaves and fruit of plum trees, as an example of higher plants (12), only small amounts of radioactivity entered the pectic acids, which are considered to be metabolically rather inactive. It has been suggested on the basis of their seasonal variations that mannitol and alginic acid are direct products of photosynthesis in brown algae (13), and the present work amply confirms this view. Thus alginic acid cannot be considered analagous to pectic acid in higher plants, for although it may play an important role as a structural element, it is also a major insoluble storage compound for photosynthetic carbon. Black (8) has postulated fucoidin as the main storage carbohydrate of Fucaceae, on the basis of its seasonal variations. It is obvious from Table VI that although this function is mainly filled by alginic acid, fucoidin also plays a considerable role.

As is seen in Table IV, the sample in experiment 5 assimilated 108 mg. of CO_2 containing 29.5 mg. carbon, and a total activity of 90 μc. Thus the specific activity of carbon taken in was $90/29.5 = 3.05$ μc. per mg. Hence the 10 μc. of C^{14} in alginic acid (Table VI) represented 3.3 mg. of newly synthesized alginic acid carbon, or 8 mg. of new alginic acid. The average dry weight of a number of similar samples of *F. vesiculosus* was determined, and found to be 300 mg. Thus the amount of radioactive alginic acid synthesized during the experiment was 2.6% of the dry weight. From the data of Macpherson and Young (13, Fig. 4), the alginic acid content of *F. vesiculosus* from the Halifax area varies from a low of about 8% in the summer to a high of 16% in winter, or a seasonal variation of about 8%. The alginic acid synthesized in the present sample during 30 hours of photosynthesis is thus equal to one third of the total variation over one year. Alginic acid might also have been formed in this sample from non-radioactive carbon during the same period, so that the figure for total alginic acid formed during the experimental time is probably considerably larger than that calculated for radioactive alginic acid. The conclusion which follows is that alginic acid is in a state of active metabolism in *F. vesiculosus*, undergoing breakdown and resynthesis at rates comparable to those of starch in land plants. The annual fluctuations which have been observed in the content of alginic acid are probably due not to the tendency of the plant to store more or less alginic acid but to seasonal differences in the rates of its synthesis and breakdown.

An interesting conclusion may be drawn from the data in Table V obtained following procedure B. It will be seen that the first hydrolysis, done with *N* HCl at 100° C. for 3 hours, released both xylose and fucose, among other sugars. The second, more severe, hydrolysis released more fucose, but no more xylose. The first hydrolysis was more than sufficient to break down fucoidin, yet more fucose was released on more severe hydrolysis. This was not due to incomplete extraction, since xylose was not present in the second

hydrolyzate. In a separate experiment to be reported in detail later, in which fucoidin had been thoroughly extracted from a similar sample of weed by the procedure of Black *et al.* (10), two similar hydrolyses of the residue gave the same results: xylose was all removed from the residue by the first hydrolysis, but considerable fucose appeared in both the first and the second hydrolyses, in spite of the extraction of fucoidin. The conclusion is that fucose is present in two forms in the plant: one as the readily hydrolyzed, water-soluble fucoidin, and the other as an insoluble compound much more resistant to acid hydrolysis. This latter may be important as a structural element in the plant, while easily hydrolyzable fucoidin may well serve as a reserve carbohydrate. It is of interest to note that whatever procedure they tried, Black *et al.* (10) were never able to remove more than 80% of combined fucose as fucoidin.

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METABOLIC CHANGES IN DETACHED WHEAT LEAVES FLOATED ON BENZIMIDAZOLE AND THE EFFECT OF THESE CHANGES ON RUST REACTION[1]

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Abstract

Stem-rust-infected leaves of resistant (var. Khapli) and susceptible (var. Little Club) wheats were detached and floated on water and benzimidazole, with and without glucose. Khapli leaves were susceptible on water, resistant on 40 p.p.m. benzimidazole, susceptible on 40 p.p.m. benzimidazole with 1% glucose, and resistant on 60 p.p.m. benzimidazole with 1% glucose. Little Club leaves were susceptible in all treatments. Khapli leaves subjected to treatments that increase the endogenous substrate levels, such as floating on water for 2 to 4 days or spraying with DDT 2 or 3 days before detachment, were susceptible on 40 p.p.m. benzimidazole and resistant or partially resistant at higher concentrations.

The progressive decrease in protein of detached leaves on water was interrupted on transfer to benzimidazole, after which some resynthesis of protein took place with a corresponding decrease in the soluble nitrogen fraction. Detached leaves on benzimidazole with 1% glucose had more soluble nitrogen than leaves on benzimidazole alone. There was more alcohol-soluble carbohydrate in leaves floated on water than in leaves on benzimidazole.

Introduction

Detached leaf culture has been used in many studies of obligate parasites (see review by Yarwood (20)). Although this method has certain advantages, a serious disadvantage is the early death of some leaves (4). In the cereal rusts, a second disadvantage is the fact that the rust reactions obtained are often not characteristic for the variety of plant in question (2). The life of detached leaves is prolonged, in some cases, where callus tissue is formed (19) or, in other cases, where rooting takes place (3). With wheat, in which neither of these occurs, it was reported in a preliminary note that detached leaves can be maintained for relatively long periods when floated on benzimidazole or kinetin solutions (12). When further studies showed that with appropriate concentrations of these chemicals typical rust reactions could be obtained, it was obvious that the method would be useful in studies of host–parasite relations.

The relation between supply of substrate and the development of the rust has been emphasized by several investigators (6, 14, 15, 16, 17). Detached leaves are particularly useful for such studies, and this paper describes some of the effects of benzimidazole on the metabolism of detached leaves, and the accompanying effects on rust development.

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Contribution No. 1 from the Canada Department of Agriculture Research Laboratory, Winnipeg, Manitoba.
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Methods

Detached Leaf Culture

Unless otherwise stated, the first leaves of 7- to 8-day-old wheat seedlings were inoculated with race 15B of *Puccinia graminis* (Pers.) f. sp. *tritici* (Erikss. & Henn.), incubated for 24 hours, and allowed to dry. Leaves were detached 1 day after inoculation and floated on 20 ml. of solution in petri dishes. Alternatively, the dishes were tilted with approximately one inch of the bases of the leaves in the test solution. An increase of 10 p.p.m. in benzimidazole concentration was required in this case to get the same effect as when the leaves were floated on the solutions.

Rust development on detached leaves of susceptible varieties was normal when they were kept in light of medium intensity. The solutions remained free of contaminating microorganisms when leaves were kept on benzimidazole solutions without added carbohydrates.

Detached leaves for analytical studies were floated on 250 ml. of solution in glass moist chambers with a diameter of 25 cm.

Nitrogen Determinations

Fresh leaf material was weighed, cut into ½-in. pieces, and exhaustively extracted with 80% ethanol. The extracts were combined and evaporated in an air stream with moderate heat. The residue was taken up in water, centrifuged, and used for determination of "soluble" nitrogen. This fraction thus included only the water-soluble fraction of the alcohol extract. Dried samples of the alcohol-extracted tissue were used for determination of insoluble nitrogen. Nitrogen determinations were made in duplicate by use of a "semimicro" modification of the procedure given in (1).

Carbohydrate Determinations

Fresh leaves were weighed, cut into ½-in. sections, dropped into boiling 80% ethanol, and refluxed for 30 minutes. The extraction was repeated with a second volume of ethanol and the two extracts were combined. The ethanol was removed in an air stream with moderate heat and the aqueous extract cleared with zinc sulphate (10), centrifuged, and made up to a standard volume. Total soluble carbohydrates in solutions were determined by use of an anthrone method (22).

Results

Effect of Benzimidazole on Rust Development

Khapli leaves, normally resistant to race 15B of stem rust, became susceptible when detached and floated on water but remained resistant when floated on 40 p.p.m. benzimidazole. The same results were obtained with Colotana 266/51 inoculated with race 15 of leaf rust (*Puccinia triticina* Erikss.). Little Club, which is susceptible to both of these rust races, remained susceptible when detached leaves were floated on water and on 40 p.p.m. benzimidazole.

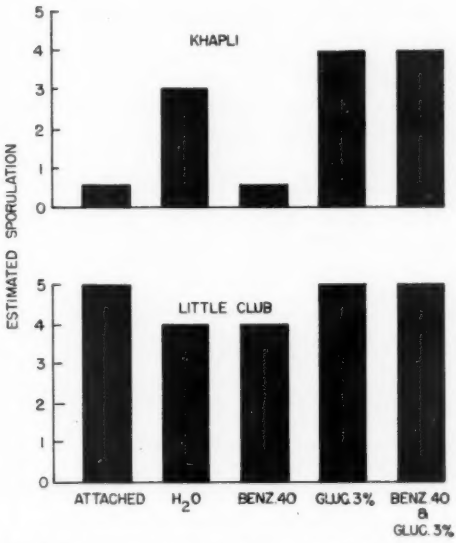

FIG. 1. Rust development on detached leaves of Little Club and Khapli wheats floated on water, benzimidazole (40 p.p.m.), glucose (3%), and benzimidazole (40 p.p.m.) with 3% glucose, compared with that on intact leaves.

When glucose was added to the benzimidazole solutions increased sporulation on the detached leaves was observed. Results of a typical experiment are given in Fig. 1, where visual estimates of the degree of sporulation on treated plants are compared with those obtained with normal intact plants. It is particularly interesting that Khapli, which is normally resistant, and resistant on benzimidazole, was susceptible on benzimidazole plus glucose. Further studies on the glucose–benzimidazole interaction were carried out, and it was found that the reversal in rust reaction, obtained by adding 1% glucose to the solutions, was prevented when the concentration of benzimidazole was increased. Figure 2 shows this for the stem rust reaction on the variety Khapli, and similar results were obtained with leaf rust on Colotana 266/51. It is interesting that increased concentrations of benzimidazole, which in this special case prevent the expression of a susceptible reaction, have little effect when used on leaves that are normally susceptible, even when concentrations up to 100 p.p.m. are used. Sporulation on normally susceptible leaves may be decreased at the higher concentrations of benzimidazole but the reaction remains typically susceptible.

Changes in protein and soluble nitrogen levels following detachment have been studied. Protein breakdown and accumulation of amino acids and amides occurred on water but not on benzimidazole (12). To determine the effect of different nitrogen levels on rust development, inoculated leaves were floated on water for varying periods before transfer to benzimidazole solutions. Rust

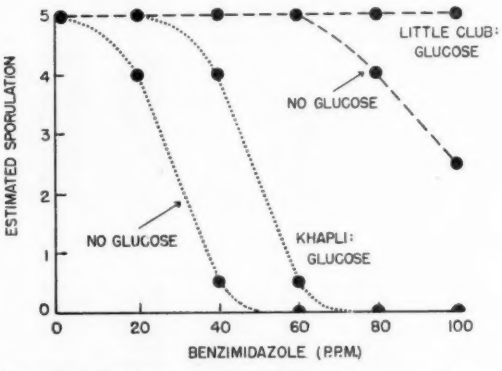

FIG. 2. Effect of benzimidazole concentrations, with and without glucose, on rust development on detached leaves of Khapli and Little Club wheats.

TABLE I

RUST REACTIONS ON DETACHED INFECTED KHAPLI LEAVES FLOATED ON WATER FOR VARYING PERIODS BEFORE TRANSFER TO BENZIMIDAZOLE SOLUTIONS

Days on water	Days on benz.	Benzimidazole concentrations (p.p.m.)			
		20	40	60	80
0	12	++++*	+	+	+
1	11	++++	++	+	+
2	10	++++	+++	+	+
3	9	++++	+++	++	+
4	8	++++	++++	+++	+

*These units have been used to express rust development since it is often difficult to determine reaction types on detached leaves. In general, ++++ is roughly equivalent to a type 4 and + to a type 1 reaction.

reactions, recorded on the 12th day following inoculation (Table I), showed that, with increasing time on water, increased concentrations of benzimidazole were needed to bring about the expression of the normal, resistant reaction.

In other experiments the leaves were inoculated after treatment, instead of before. Table II lists the results of one such experiment, in which detached leaves were floated on water or glucose solutions before inoculation and transfer to benzimidazole. With a 6-day treatment before inoculation and transfer to benzimidazole there was good rust development, even at the high concentrations of benzimidazole. The rapid deterioration which normally takes place in detached leaves was interrupted on transfer to benzimidazole, permitting continued growth of the rust.

Spraying with DDT is known to break down the normal resistance of Khapli and render it susceptible to stem rust (6, 8). Rust development on Khapli leaves on benzimidazole following DDT treatment is shown in Table III. Induced susceptibility was maintained on the lower concentrations of benzimidazole, and reversed or partially reversed at the higher concentrations.

TABLE II

RUST DEVELOPMENT ON DETACHED KHAPLI LEAVES GIVEN VARIOUS TREATMENTS BEFORE INOCULATION AND TRANSFER TO BENZIMIDAZOLE SOLUTIONS

Preinoculation treatment		Benzimidazole concentrations (p.p.m.)			
		20	40	60	80
Water	3 days	++++	+++	++	+
1% glucose	3 days*	++++	++++	+++	+++
Water	6 days	++	++++	++++	++++
1% glucose	6 days*	++	++++	++++	++++

*Postinoculation treatment: benzimidazole in 1% glucose.

TABLE III

RUST DEVELOPMENT ON KHAPLI LEAVES SPRAYED WITH DDT BEFORE DETACHING AND FLOATING ON BENZIMIDAZOLE SOLUTIONS

Interval in days between DDT application and detaching	Benzimidazole concentrations (p.p.m.)			
	40	60	80	100
0	+	+	+	+
2	+++	++	++	+
3	+++	+++	++	++

In summary: normally resistant leaves when detached and placed on water become susceptible; on benzimidazole (40 p.p.m.) they remain resistant. The resistance obtainable on benzimidazole is reversed if glucose is added, or if leaves are pretreated by floating them on water or on glucose, or by spraying with DDT, but this reversal can be prevented by increasing the concentration of benzimidazole to levels which, when applied to leaves normally susceptible, have no deleterious effect on rust development.

Effect of Benzimidazole on Metabolism of Detached Leaves

The changes in nitrogen content of the alcohol-soluble and -insoluble fractions taken from detached leaves have been described (12). There was an increase in soluble nitrogen and a decrease in the insoluble nitrogen fraction of detached leaves on water, whereas on benzimidazole there was little change in either of these fractions. The time curves given in Fig. 3 show the effect on nitrogen levels of maintaining leaves on water for different periods before transferring them to benzimidazole. The nitrogen values were obtained from duplicate lots of five leaves each. The insoluble fraction is expressed as a percentage of "total" nitrogen, that is, of the sum of the soluble and insoluble fractions. The curves show that the downward drift in the insoluble nitrogen fraction, which normally occurs in detached leaves on water, was interrupted on transfer to benzimidazole, and insoluble nitrogen was held at a new level. The curves show very clearly that protein synthesis in detached leaves was promoted by benzimidazole.

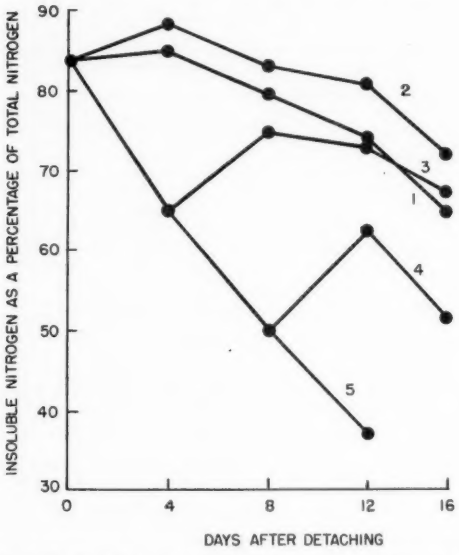

FIG. 3. The effect of benzimidazole on the nitrogen metabolism of detached leaves of Khapli wheat. Symbols: 1, attached leaves; 2, sixteen days benzimidazole; 3, four days water, twelve days benzimidazole; 4, eight days water, eight days benzimidazole; 5, twelve days water.

Table IV shows the effects on nitrogen levels in detached Little Club leaves when glucose (1%) was added to the benzimidazole solutions. Curves showing changes in the ratio of the soluble and insoluble fractions are given in Fig. 4. For this experiment, healthy and inoculated leaves were floated on the following solutions: water, glucose (3%), benzimidazole (40 p.p.m.), and benzimidazole (40 p.p.m.) plus glucose (3%). The leaves were transferred to fresh solutions each day in order to reduce contamination. One hundred and fifty

TABLE IV

CHANGES IN NITROGEN LEVELS* OF HEALTHY AND RUST-INFECTED LEAVES OF LITTLE CLUB WHEAT FLOATED ON WATER AND ON BENZIMIDAZOLE (40 P.P.M.) IN THE PRESENCE AND ABSENCE OF GLUCOSE

Treatment		Days after detaching					
		6		9		12	
		Soluble	Insoluble	Soluble	Insoluble	Soluble	Insoluble
H_2O	Healthy	0.30	0.63	0.36	0.42	—	—
	Rusted	0.30	0.75	0.48	0.52	—	—
Glucose (3%)	Healthy	0.28	0.79	0.38	0.46	—	—
	Rusted	0.42	0.78	0.28	0.57	—	—
Benz. (40 p.p.m.)	Healthy	0.23	0.94	—	—	0.12	0.91
	Rusted	0.17	1.01	—	—	0.14	0.83
Benz. (40 p.p.m.) and glucose (3%)	Healthy	0.19	0.92	—	—	0.24	0.86
	Rusted	0.20	1.06	—	—	0.14	0.86

*Alcohol- and water-soluble nitrogen and alcohol-insoluble nitrogen expressed as mg. nitrogen/150 disks.

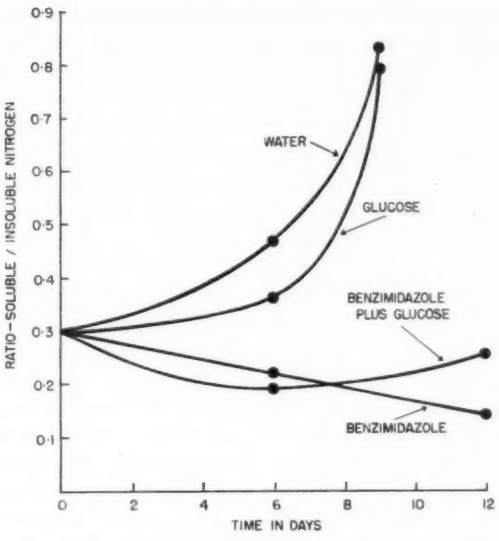

FIG. 4. The effects of 1% glucose on the nitrogen levels of leaves of Little Club wheat floated on water and on 40 p.p.m. benzimidazole.

leaf disks, taken with a 3-mm. disk cutter, were used for each analytical sample. The curves of Fig. 4 illustrate again the effect of benzimidazole in stabilizing protein levels in detached leaves. Although the addition of glucose did not bring about any profound change in nitrogen levels, there was more soluble nitrogen after 12 days than when leaves were treated with benzimidazole alone.

Levels of total alcohol-soluble carbohydrates of detached Khapli leaves with and without benzimidazole are listed in Table V. During the period of treatment the leaves on water yellowed progressively, but the sugar levels were appreciably higher than for those on benzimidazole. Since it has been found that CO_2 fixation continues on benzimidazole (Dr. D. T. T. Wang, personal communication), the differences here recorded indicate increased utilization of carbon, presumably in synthetic reactions, in the benzimidazole-treated leaves.

TABLE V

TOTAL ALCOHOL-SOLUBLE CARBOHYDRATE LEVELS (EXPRESSED AS % OF FRESH WEIGHT) IN DETACHED KHAPLI LEAVES FLOATED ON WATER AND ON BENZIMIDAZOLE

Days after detaching	Water	Benzimidazole, 40 p.p.m.
7	3.6	2.0
11	2.7	0.7

Results given in Table V were confirmed in subsequent work by use of paper partition chromatography. Compared with leaves on benzimidazole, those floated on water contained considerably larger amounts of sucrose, glucose, and fructose, and in addition, large amounts of what appear to be oligosaccharides with R_F-values smaller than that of sucrose.

In summary, the analytical work shows that benzimidazole prevents the downward drift in alcohol-insoluble nitrogen content which normally occurs in detached leaves. When water pretreatment is followed by benzimidazole the downward drift is stopped and the insoluble nitrogen held at a new level. Addition of glucose to the benzimidazole solutions results in an increase of nitrogen in the alcohol-soluble fraction. Benzimidazole-treated leaves contain less alcohol-soluble carbohydrate than leaves floated on water alone.

Discussion

With relatively few exceptions, there is some development of the rust mycelium within the tissues of the infected wheat leaf. When this development is limited and sporulation is sparse, the host is usually termed resistant; when mycelial development is more extensive and sporulation abundant, the host is susceptible. A dividing line has been arbitrarily set in order to delimit the two classes "resistant" and "susceptible", even though there is a gradual and continuous range between the two extremes.

For any specific host–rust combination there is a characteristic norm for the rust reaction obtainable, but there is also a range of variation about this norm, attributable to environment, as there is with any biological system. With certain host–rust combinations, however, large changes in rust reaction may be obtained with relatively small changes in external environment (7). A special case of variation in rust reaction, from highly resistant to completely susceptible, is usually seen when infected resistant leaves are detached and floated on water. Rust development then resembles that on completely susceptible leaves.

In attempting to explain these variations in rust reactions, both large and small, in terms of the metabolism of the infected leaf, there appear to be two possibilities. Increased rust development may be due to the removal of inhibitory substances, or to an increase in substances essential to the rust that were previously insufficient for its development. The second of these possibilities seems to us, on the basis of our present information, to be the more reasonable of the two.

A characteristic feature of detached leaves on water is a decrease in protein and a concomitant increase in amino acids (12, 21). Alcohol-soluble carbohydrates also tend to increase in detached leaves. Associated with these changes is a breakdown in rust resistance. In contrast, leaves floated on benzimidazole show no appreciable changes in levels of protein, amino acid, or alcohol-soluble carbohydrates. Neither is there any breakdown in rust resistance. Since benzimidazole has no effect on rust development in susceptible leaves, its action in maintaining the resistance of resistant leaves must

result from its effects on the metabolism of the host. A possible explanation for the difference in rust reaction in the two situations is that substrate, normally limiting at infection loci in intact resistant plants, or in detached resistant leaves on benzimidazole, is no longer limiting in those treatments which result in a susceptible reaction.

The availability of substrate at the site of infection, and its importance in determining the disease reaction, have been studied by previous workers. Shaw and Samborski (17) showed that metabolites move to the infection sites from other uninfected areas of the leaf, and that the accumulation of substrate at the infection loci is dependent on aerobic respiration. Samborski and Shaw (13, 14, 15) suggested that there is competition for substrate between the infected areas and other regions of high metabolic activity in the plant. They suggested also that the final disease reaction at any infection locus results from the interplay between the supply of individual metabolites, either through synthesis or transport, on the one hand, and the utilization of these metabolites, either by the host cells or the parasite, on the other. Shaw and Hawkins (16) showed that indoleacetic acid (IAA) levels in infected susceptible leaves are higher, and in infected resistant leaves lower, than in uninfected control leaves. They considered that higher IAA levels would favor the mobilization of metabolites at the infection loci in the infected susceptible leaves. Daly and Inman (5) found higher levels of growth-promoting substances in safflower hypocotyls following infection and considered that this change would result in greater abundance of substrate and thus provide conditions favorable to mycelial development.

The results of the present study are consistent with the view that rust reactions are determined by the availability of substrate at the infection loci, and that this in turn is influenced by the level of IAA. Since all the nutritional requirements of the rust are supplied by the host, an insufficient level of any essential compound would inhibit rust development. This is implied in the concept of limiting substrate. In our experiments, where conditions are made unfavorable for rust development, the analytical results indicate that soluble nitrogen is the limiting factor.

Treatments that permit rust development in normally resistant leaves also result in an increase in soluble substrate (6, 14). With DDT treatment, higher concentrations of benzimidazole are needed to maintain the normal resistant reaction. Benzimidazole stimulates protein synthesis in detached leaves (Fig. 3). If the rate of induced synthesis is dependent on the concentration of benzimidazole used, an increase in concentration would result in a more rapid depletion of nitrogen. The availability of soluble nitrogen could, in such a case, determine the rate of development of the fungus.

The addition of glucose to 40 p.p.m. benzimidazole results in abundant rust development; this treatment also results in an increase in the level of soluble nitrogen (Fig. 4). With Khapli leaves floated on 1% glucose, a relatively slight increase (from 40 to 60 p.p.m.) in the concentration of benzimidazole is sufficient to reverse the rust reaction (Fig. 2). In this case it is unlikely that

glucose is the factor limiting rust development, but rather that protein synthesis in the host cells, stimulated by benzimidazole, causes a drop below critical values for one or more amino acids that are essential to rust development. Chromatographic studies show that the increase in concentration of benzimidazole from 30 to 60 p.p.m., which confers resistance in the presence of 1% glucose, also results in a decrease in certain amino acids which are normally present in small quantities (unpublished data).

If it is accepted that amino acids are essential to rust development, attention must be directed to the possibility that rust races may differ in their specific amino acid requirements. Zscheile and Murray (23) concluded that genes for resistance to rust, bunt, and Hessian fly had no apparent effect on the levels of specific amino acids in wheat ovules. However, the amino acid composition is not the same in all parts of the plant. Differences between mature and actively growing tissue in levels of individual amino acids have been demonstrated (18). In other work, it was shown that mature tissue of wheat was less susceptible to rust than immature tissue (11). Since it is those amino acids which are present in very small amounts that are most likely to be critical in the development of the rust (and some are known to be present in very low concentrations (9)), the evaluation of their importance will require careful and exhaustive studies.

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OCCURRENCE OF TRAMETES SEPIUM IN INDIA[1]

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Abstract

Trametes sepium Berk. is reported for the first time in India from the temperate regions in the Western Himalayas, on structural timber of *Cedrus deodara*. The sporophores of the fungus as they occur in India and the cultures are described. The identity of the fungus was confirmed by interfertility tests between the Indian and American collections of the fungus.

Trametes sepium Berk. is recorded from dead wood, usually of hardwood (deciduous) trees, occasionally of coniferous trees. It is reported from North America by Nobles (3) and Overholts (4) and from Europe under the name *Trametes* (*Coriolellus*) *albida* by Bourdot and Galzin (2) and Bondarzew (1). It was recently collected on bridge timbers of deodar (*Cedrus deodara*) in Chakrata in the temperate Himalayas. This is the first record of the fungus from India.

The following description of the fungus is based on this collection.

Sporophore annual, sessile, effuso-reflexed (Fig. 1) or resupinate (Fig. 2), often imbricate, flexible when fresh, firm corky when dry; pileus 0.3–3.2 cm. long, 0.3–1.0 cm. wide, and 0.2–0.4 cm. thick, the surface short pubescent when fresh, becoming nearly glabrous later, white when fresh, becoming brown on drying, "pale ochraceous salmon", "Saccardo's umber", and "sepia" (Ridgway (5)), particularly near the margin, zonate, the margin thick, obtuse; context white to "pale ochraceous salmon", up to 0.2 cm. thick, the hyphae hyaline, thick-walled, with lumen narrow or nearly obliterated (Fig. 6), 3.0–6.0 μ in diameter; hymenial surface white, "pinkish buff", "light ochraceous buff", the pores averaging 1 to 2 per mm., circular to angular (Fig. 2), with edges moderately thick, the tubes 1–3 mm. long, the hyphae as in the context, occasionally with clamps, or partly thick-walled, branched, with clamp connections common (Fig. 7), 3.0–5.0 μ in diameter; basidia clavate, 6.0–8.0 μ diameter, with long sterigmata; basidiospores hyaline, smooth, thin-walled, cylindric or cylindric-ellipsoid (Fig. 9), 7.0–11.0 $\times$ 3.0–4.5 μ.

The fungus produces a brown rot in the wood, in which cracks appear, containing white mycelial mats (Fig. 3).

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Joint contribution from the Forest Pathology Branch, Forest Research Institute, Dehra Dun, India, and the Botany and Plant Pathology Division (Contribution No. 1692), Science Service, Canada Department of Agriculture, Ottawa, Ontario.
[2]Dehra Dun, India.
[3]Ottawa, Ontario.

A culture of the fungus was isolated and the following description of its growth and hyphal characters was prepared.

GROWTH CHARACTERS.—Growth moderate, the radial growth on malt agar in the dark, at 25° C., is 2.0–2.5 cm. in 1 week. The advancing zone hyaline, even; the mat appressed, downy, changing to chalky white, with pebbled areas which develop pored fruiting surfaces (Figs. 4 and 5) after 4 to 6 weeks. Reverse unchanged. Odor faint. On gallic and tannic acid agars, no diffusion zones, the radial growth 1.2–1.6 cm. on the former and trace to 0.5 cm. on the latter. On gentian violet agar, growth moderate and medium not discolored.

HYPHAL CHARACTERS.—*Advancing zone*: (i) hyphae hyaline, partly thick-walled, branched, septate, with clamp connections (Fig. 10), 2.0–5.0 μ in diameter; (ii) thick-walled hyphae with irregular lumen (Fig. 11). *Aerial mycelium*: (i) hyphae as in advancing zone; (ii) hyphae with irregularly thickened walls, branched, septate with clamp connections, and irregular lumen (Fig. 12), up to 6.0 μ diameter. *Hyphae from pebbled area* (initial stage of fruit body): (not easily separable) (i) hyphae with irregularly thickened walls (Fig. 13); (ii) fiber hyphae numerous, refractive, usually with solid walls, sometimes with narrow or nearly obliterated lumen, occasionally branched, or septate with clamp connections, sometimes swollen (Fig. 14), 2.0–3.3 μ diameter. *Submerged mycelium*: hyphae with clamp connections, as in aerial mycelium, sometimes breaking at septa. *Fruit body* (formed readily in culture) (Figs. 4 and 5): hyphae as in fruit body in nature; basidia clavate, 6.0–8.0 μ diameter; basidiospores hyaline, cylindric to cylindric-ellipsoid, 6.0–10.0×3.0–4.0 μ, the basidia and basidiospores similar to those found in natural fruit bodies, except for the spores being slightly smaller and narrower. Nobles (3) stated that "*T. sepium* has not produced basidiospores in culture although pored fruiting surfaces have developed". In our cultures of the fungus perfect fruit bodies with basidia and basidiospores develop readily.

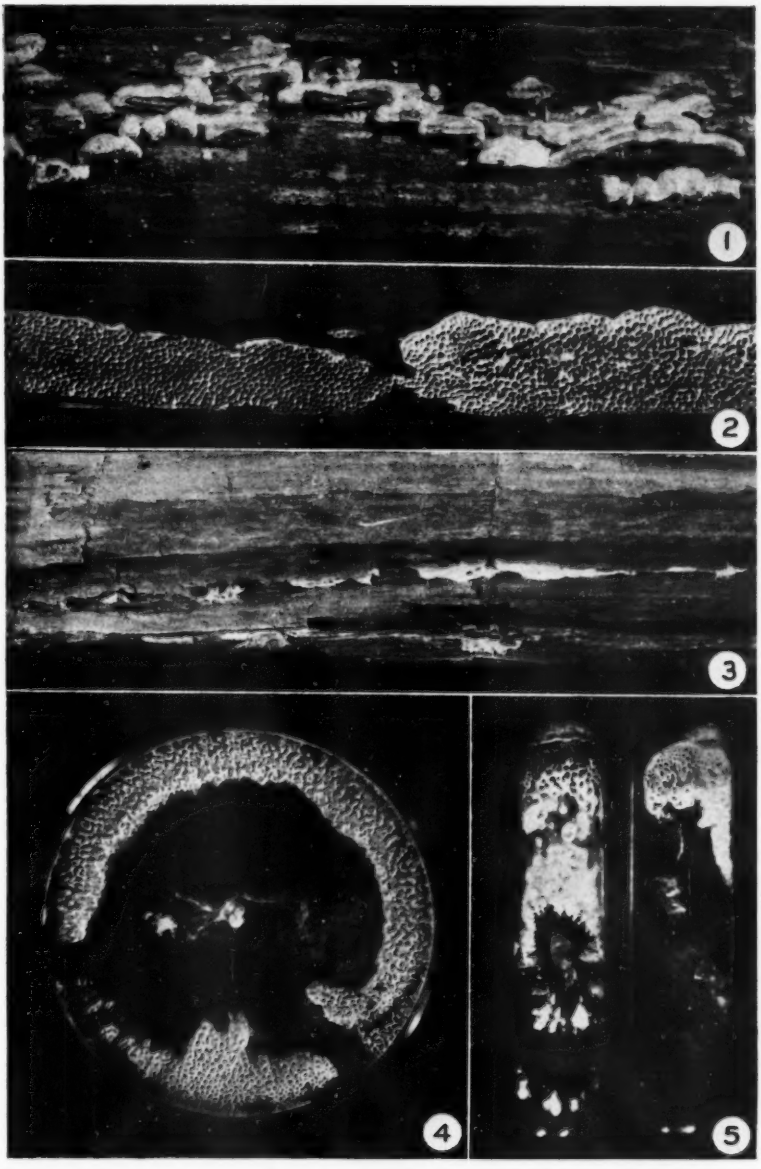
TABLE I

SOURCE OF CULTURES USED IN INTERFERTILITY TESTS

DAOM No.	Host	Locality	Received from:
52343	—	St. Michaels, Md., U.S.A.	Beltsville Forest Disease Laboratory (BPI 103468)
52344	*Acer rubrum*	Portsmouth Co., Va., U.S.A.	Beltsville Forest Disease Laboratory (BPI 103723)
52345	*Liriodendron tulipiferae*	Portsmouth Co., Va., U.S.A.	Beltsville Forest Disease Laboratory (BPI 103730)
52825	*Cedrus deodara*	Chakrata, Himalayas, India	Forest Research Institute, Dehra Dun, India (No. 376)

Single basidiospores from a fruit body formed in the culture of *Trametes sepium* from India were isolated by the junior author and interfertility tests were carried out between this Indian isolate and monosporous cultures isolated from three sporophores collected on broad-leaved trees in the United States. The collection data for the latter are listed in Table I. In the interfertility tests, the results of which are shown in Figs. 15, 16, and 17, more or less complete fertility was observed. In this way, the identification of the Indian specimen as *Trametes sepium* by the senior authors was confirmed by the junior author by means of interfertility tests. Thus, the occurrence of *Trametes sepium* in India on a coniferous host was definitely established.

FIG. 1. Sporophore of *Trametes sepium*, effuso-reflexed form (×1).
FIG. 2. Sporophore of *T. sepium*, resupinate form (×1.3).
FIG. 3. Rot associated with *T. sepium*, showing mycelial mats in crevices of wood (×1.6).
FIG. 4. Culture of *T. sepium*, 5 weeks old, showing poroid fruiting areas (×0.7).
FIG. 5. Culture of *T. sepium*, 5 weeks old, showing poroid fruiting areas (×0.9).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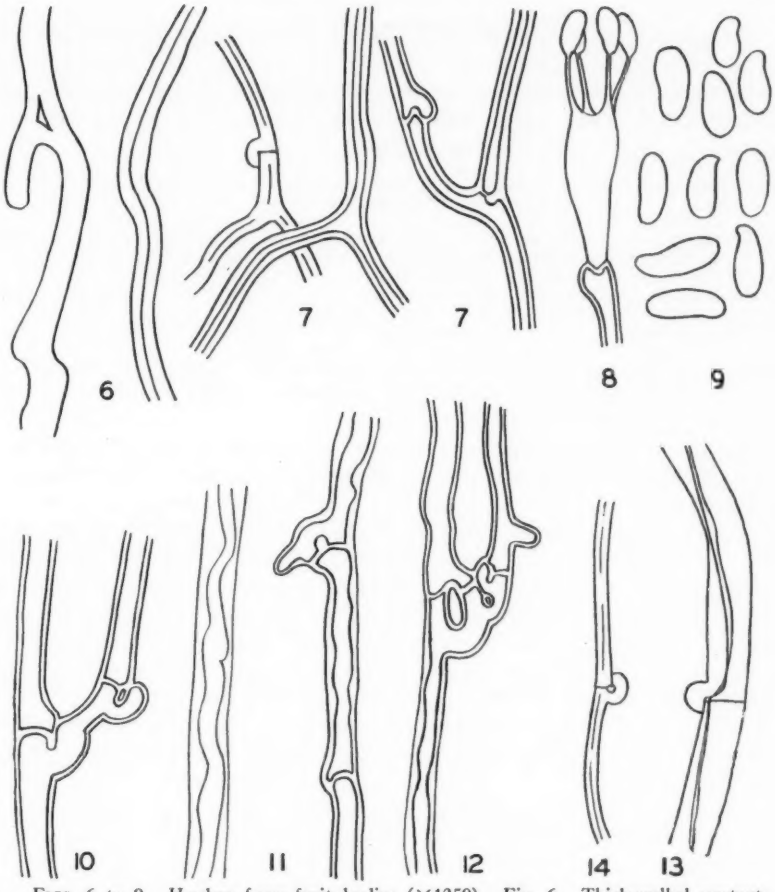

FIGS. 6 to 9. Hyphae from fruit bodies (×1250). Fig. 6. Thick-walled context hyphae. Fig. 7. Thick-walled hyphae from hymenium. Fig. 8. Basidium. Fig. 9. Basidiospores.

FIGS. 10 to 14. Hyphae from cultures (×1250). Fig. 10. Thick-walled hyphae, septate with clamp connections. Figs. 11 and 12. Thick-walled hyphae with irregular lumen. Fig. 13. Hypha with irregularly thickened walls. Fig. 14. Thick-walled fiber hyphae, with very rare clamp connections.

52343	52825	1	2	3	4
	1	+	+	+	+
	2	+	+	+	−
	3	+	+	+	−
	4	+	+	+	+

15

52344	52825	1	2	3	4
	1	+	+	+	+
	2	−	+	+	+
	3	+	+	+	+
	4	−	−	+	−

16

52345	52825	1	2	3	4
	1	−	−	−	+
	2	−	+	−	−
	3	+	−	+	−
	4	+	−	+	+

17

FIGS. 15 to 17. Results of pairing of monosporous mycelia, the (+) sign indicating the presence of hyphae with clamp connections, the (−) sign, their absence.

Acknowledgments

The authors wish to thank Dr. H. H. McKay and Mrs. F. Lombard of the Beltsville Forest Pathology Laboratory, Forest Service, United States Department of Agriculture, for providing the monosporous cultures of *Trametes sepium* that were used in interfertility tests.

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CYTOLOGICAL STUDIES OF VENTURIA INAEQUALIS[1]

J. B. JULIEN[2]

Abstract

A cytological study of *Venturia inaequalis* has revealed that only one meiosis occurs in the ascus, and that the haploid number of chromosomes is seven. Most asci originate through typical hooks, but in a number of perithecia these croziers are absent, and large budding cells give rise to small ascus-shaped structures. Variations in nuclear stainability were observed among perithecia at the same stage of maturity. Attempts were made to characterize the seven chromosomes.

Introduction

Venturia inaequalis (Cke.) Wint., the ascogenous stage of *Fusicladium dendriticum* (Wallr.) Fckl., causes the well-known apple scab disease.

The initiation of the ascogenous stage has been worked out by Killian (8, 9) and Frey (4), who agree that there is no nuclear fusion in the ascogonium. However, the two authors differ as to the origin of the ascus. Killian (9), supported by Backus and Keitt (1), states that the ascus is formed from the penultimate cell of a crozier, whereas Frey (4) believes that certain large cells of the ascogonium, by budding, give rise to a number of small ascogenous cells, some of which develop directly into asci without the typical crozier formation.

The potentialities of this fungus as a genetical tool have been recognized since Keitt and his co-workers (7, 12) showed it to be heterothallic, and since Shay and Williams (13) demonstrated the existence of physiological races. Boone (2) has found genetic markers for four chromosomes. Day *et al.* (3) mention that Boone and Keitt (unpublished) have obtained genetic evidence indicating at least six linkage groups.

The haploid number of chromosomes has been claimed to be four to six by Backus and Keitt (1) in 1940, and seven by Day *et al.* (3) in 1956.

The present study was begun in 1953, and its primary purpose was to obtain an accurate count of the number of chromosomes of *Venturia inaequalis*, and to follow the chromosome behavior throughout the development of the ascus.

Materials and Methods

Perithecia were isolated from overwintered apple leaves collected at intervals of a few days in the spring. Fruiting bodies fixed shortly after collection (within 1 to 3 days) showed a wider range of stages of nuclear division than

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Joint contribution from the Department of Botany, University of Toronto, Toronto, Ontario, and from the Botany and Plant Pathology Division, Science Service, Canada Department of Agriculture, Ottawa, Ontario (Contribution No. 1620). Part of a thesis presented to the University of Toronto in 1957 in partial fulfillment of the requirements for the degree of Doctor of Philosophy.
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others that were kept dry for 4 months or more. Early leaf collections (mid-April) yielded large numbers of croziers; late collections (to end of May) gave more maturing or mature asci.

Perithecia were fixed in a mixture of six parts of absolute ethyl alcohol, one part of glacial acetic acid, and one part of lactic acid (14), or acetic alcohol (1:3). Following fixation, the perithecia were washed in water, hydrolyzed in *N* HCl, and dissected in acetic acid. Two per cent aceto-orcein (natural orcein from G. T. Gurr Ltd., London, and E. Gurr Ltd., London) was used to stain the preparations. To flatten the asci, the cover slips were held in position by means of special clamps while the sealing medium was drying. A description of the clamp and its use will appear in a later publication.

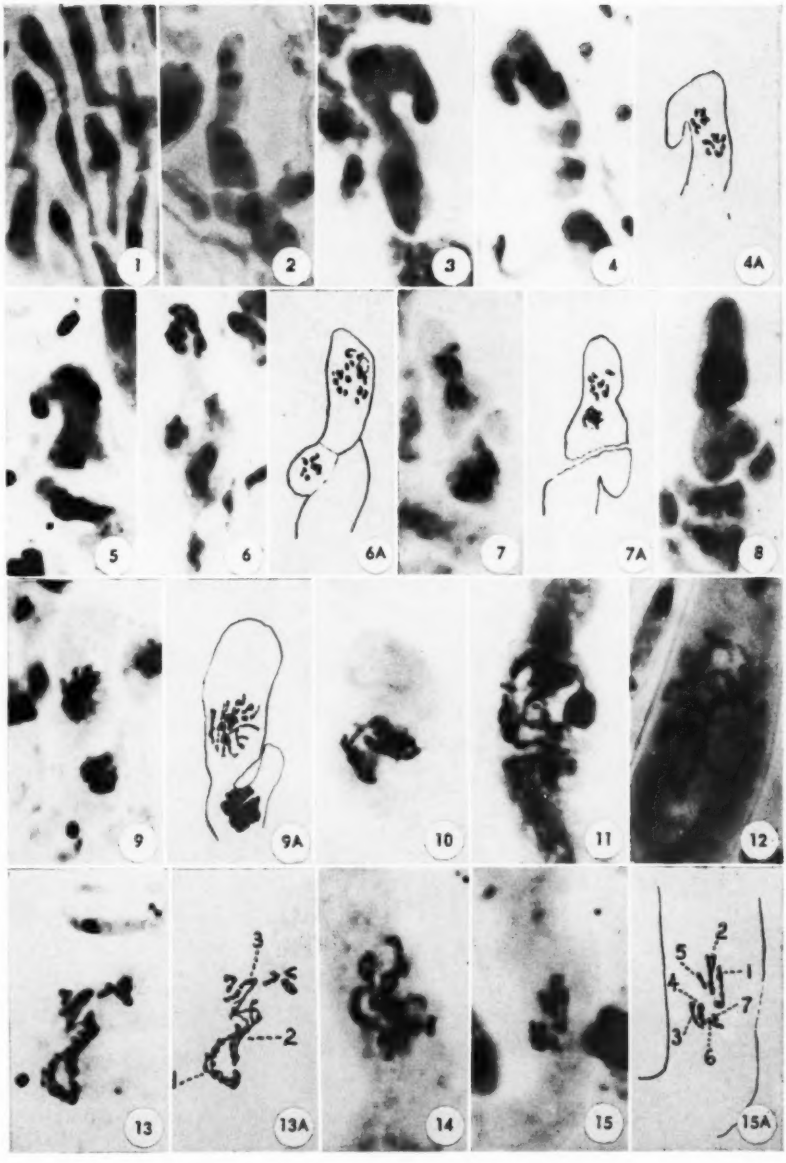
Critical studies of the preparations were made with E. Leitz Wetzlar apochromatic oil immersions 90/1.32 and 90/1.40, and a double diaphragm condenser (N.A. 1.40). The photomicrographs were taken through a No. 60 Wratten filter with a Leica 35-mm. camera, on Dupont-Safety microcopy panchromatic negative or on Kodak panatomic-X films. Photomicrographs were printed at a magnification of ×1700 except where otherwise stated. The camera lucida drawings do not always correspond exactly to what is seen in the accompanying photomicrograph because the drawings represent more than one focal plane.

Observations

The nuclei of the ascogenous hyphae, paraphyses, and vegetative hyphae differed in size and in their reaction to stain. In both the conidia and the vegetative hyphae the nuclei were very small and stained faintly. The nucleolus was not observed with certainty in the conidia or in the vegetative hyphae. Cells of vegetative hyphae, conidiophores, and conidia were uninucleate as reported by Backus and Keitt (1).

By contrast, the nuclei of the ascogenous hyphae (Fig. 2) were much larger and took slightly more stain than those of the vegetative stages just discussed. Their nucleoli were not observed except in crozier primordia. Cells of the ascogenous hyphae were either uninucleate or binucleate. The paraphyses were uninucleate and their nuclei stained intensely (Fig. 1). These nuclei were compact and slightly smaller than those of the ascogenous hyphae.

FIGS. 1–15A. All ×1700. FIG. 1. Paraphysis nuclei. FIG. 2. Ascogenous hypha showing binucleate condition. FIGS. 3 to 5. Prophase, metaphase, and telophase nuclei, respectively, in crozier. FIGS. 6, 6A. Migration of the contracted chromosomes of both nuclei into the center of the elongating subterminal cell. Note crosswall formation. The basal cell contains only one nucleus. FIGS. 7, 7A. Elongation of the subterminal cell. Seven chromosomes in the upper nucleus could be counted. FIG. 8. Fusion nucleus in the young ascus. FIGS. 9, 9A. Synapsis being not simultaneous. FIG. 10. Elongation of the bivalents. FIGS. 11, 12. Pachytene. Note the nucleolus. Chromomeres are evident and doubleness in the chromosomes is apparent. FIGS. 13, 13A. Pachytene. Note that the ascus wall has been broken allowing the spreading of the chromosomes. FIG. 14. Diplotene. Note the opening out of the chromosomes. FIGS. 15, 15A. Diakinesis. Note the Y shape of bivalent 2.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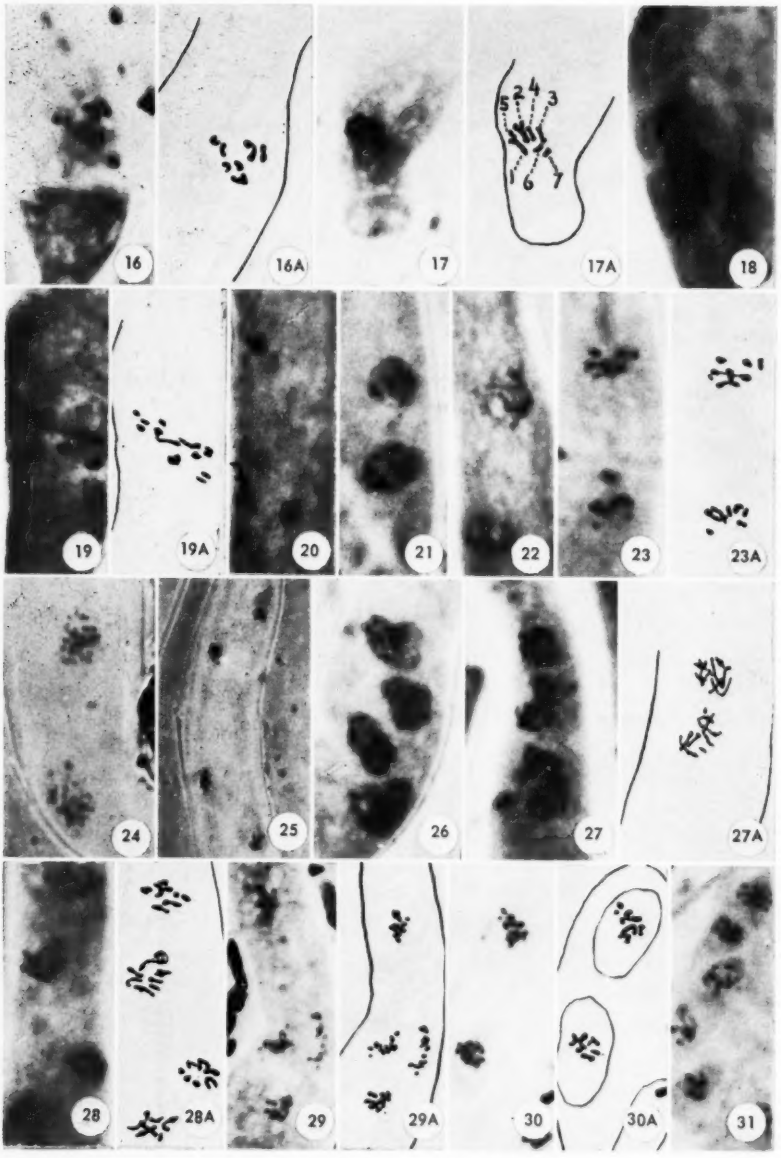
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In occasional perithecia large cells were observed which were void of croziers. It is possible that these corresponded to the ascogenous cells (ascogonia) described by Frey (4). Unfortunately the scarcity of these cells made it impossible to follow their further development.

Killian (9) noticed that ascogenous hyphae arose as lateral outgrowths from the ascogonium. The present writer observed that these ascogenous hyphae were rather irregular in shape (Fig. 2), and were distinguishable from the paraphyses by their broader and shorter cells. A few of the binucleate cells of the ascogenous hyphae developed into a crozier. The two nuclei took up positions within the crozier where they began a simultaneous mitosis.

Progressive stages in crozier development and ascus formation are illustrated in Figs. 3 to 8. As the nuclei entered prophase (Fig. 3), chromosomes became distinct and appeared to radiate from a common point near the nuclear membrane. Contraction of the chromosomes then occurred, and at the metaphase stage seven chromosomes could be counted. Since the photomicrograph did not resolve clearly the seven chromosomes a camera lucida drawing is added (Fig. 4A).

The ascus originated from the penultimate cell as described by Backus and Keitt (1). Two nonsister nuclei (Figs. 5, 6, 7, and 8) derived by the synchronous crozier mitosis fused to give the primary nucleus of the ascus.

Division I

Synapsis (Fig. 9) began soon after nuclear fusion (Fig. 8). Chromosome threads started to pair long before their maximum elongation had been attained. There was a continuous increase in size of the ascus accompanied by an increase in size of the zygote nucleus up to full pachytene (Figs. 10 to 13). As the ascus enlarged rapidly, the bivalents lengthened and structural differentiation (Fig. 10), in the form of regions of greater and less diameter and differential stainability, could be observed. The nucleolus enlarged rapidly, and reached its maximum size while the chromosomes were still elongating. The pachytene chromosomes (Figs. 11–13) were similar in appearance to those of higher organisms. At this stage, a nuclear membrane could not be demonstrated, and well-flattened preparations gave some indication of the relative length of the seven chromosomes as shown in Fig. 13A. This

FIGS. 16–31. All ×1700 except where otherwise stated. FIGS. 16, 16A, 17, 17A. Metaphase I. Bivalent 2 is Y-shaped in Fig. 17. FIG. 18. Anaphase I. Note the spindle fibers and the centrioles at each pole. FIGS. 19, 19A. Anaphase I. Twelve univalents and one bivalent (bottom center of the nucleus) can be seen. FIG. 20. Telophase I. Note the persistence of the nucleolus (arrow). FIG. 21. Interphase I. Note presence of nuclear membrane. FIG. 22. Prophase II. FIGS. 23, 23A. Metaphase II where seven chromosomes were counted in each nucleus. FIG. 24. Early anaphase II. Approximately 14 chromosomes in each of the two nuclei. FIG. 25. (×1120) Telophase II. FIG. 26. Interphase II. FIGS. 27, 27A. Late prophase III. FIGS. 28, 28A. Metaphase III. Polar view. Seven chromosomes were counted in each nucleus. Small nucleolus was observed in the second nucleus from the top. FIGS. 29, 29A. (×1120) Mid-anaphase III. FIGS. 30, 30A. Metaphase IV. Seven chromosomes were counted in the top nucleus. FIG. 31. Anaphase IV. Approximately seven chromosomes were counted in each sister nucleus.

drawing from the photomicrograph shows that there were three long bivalents, No. 1 being the longest, and four short ones, the smallest of which would be No. 7 (not marked).

After the full pachytene the chromosomes began to contract. The diplotene stage (Fig. 14) was characterized by an opening out of the bivalents. As the contraction of the chromosomes continued, the nucleus entered diakinesis, and the seven bivalents were generally individually distinct. At this stage the bivalents could be tentatively identified by size differences (Fig. 15). The numbering of the bivalents was not intended as positive identification but for convenience in reference.

The nucleolus decreased in size and as the nucleus entered metaphase the nucleolus stained very lightly. The nucleolus persisted until telophase (Fig. 20).

At metaphase the bivalents reached their maximum contraction and became closely aggregated (Figs. 16, 17). In the nucleus shown in Fig. 17, the longest bivalent was 1.5 μ. The second bivalent, as in Fig. 15 (diakinesis), frequently had its two chromosomes separated at one end. Bivalent 3, next in order of length, could also be recognized, but the other four were generally so much contracted that it was difficult to differentiate them by length.

Centrioles were observed at metaphase and early anaphase (Fig. 18) at both poles of the spindle. The bivalents were arranged at the equatorial region of the spindle, which was oriented parallel or obliquely to the longitudinal axis of the ascus. At anaphase of all four divisions, approximately 14 chromosomes could be counted, but their small size did not allow identification with the same ease as in diakinesis (Fig. 15) and metaphase (Fig. 17). Half of the chromosomes passed to each pole. Figure 19 shows 12 well-separated chromosomes and one bivalent in which the two chromosomes were still in contact and so exhibit lack of synchronization.

As anaphase proceeded the spindle elongated and the chromosomes of each group formed a compact mass; the remains of the nucleolus could still be seen between these groups (Fig. 20).

From telophase to interphase the chromosomes elongated, the nuclear membrane appeared, and finally enclosed the resting chromosomes, certain parts of which remained deeply staining as chromocenters (Fig. 21).

Division II

In the prophase of the second division, the chromomeres became evident, then the chromosomes began to contract, and the nuclear membrane disappeared (Fig. 22). At metaphase the chromosomes were again countable. Seven deeply stained chromosomes are seen in Fig. 23. This number corresponds to the number of bivalents at metaphase I, and to the number of chromosomes at metaphase of the third and fourth divisions. Although the chromosomes differed in length, it was not possible to recognize with certainty even the three longest.

Although a well-stained spindle was not observed, from the orientation of the division figures it would appear that most of the spindles were parallel to the long axis of the ascus. Occasionally, however, the spindle was oriented obliquely across the ascus.

The two nuclei divided simultaneously (Fig. 24) and spindle elongation again accompanied the anaphase movement of the chromosomes. At telophase four nuclei were present in the ascus (Fig. 25). Meiosis was completed with telophase II. The chromosomes now began to elongate and then entered the interphase (Fig. 26) in much the same way as in the first division.

The nucleolus was observed in metaphases of divisions II and III, and in prophase of division IV; it remained in evidence until late telophase of these divisions.

Divisions III and IV

The stages of the third division closely resembled those of the second division. At late prophase (Fig. 27) and at metaphase (Fig. 28), the shape and structure of the chromosomes could be easily seen. The longest chromosome of the top nucleus of the ascus in Fig. 27 measured 3 μ. Its centromere was slightly off-center.

Figure 29 shows four groups of 14 contracted chromosomes and the position of the nuclei was such that the central nonsister nuclei could pass each other in the ascus. At the end of this division a hyaline wall was laid down delimiting eight ascospores.

The fourth division, like the third, was mitotic. Chromosomes at prometaphase and metaphase (Fig. 30) resembled those seen at the same stages of the third division (Figs. 27, 28), but were more difficult to study because of their smaller size. However, in spore division such as that in Fig. 30, the seven chromosomes could be counted and their morphology conformed to that observed in the earlier division. At late anaphase (Fig. 31) the two groups of chromosomes moved to opposite ends of the spore where approximately seven chromosomes could be counted.

Following telophase a septum was laid down across the spore and finally the spore wall and the septum thickened and became greenish in color. One cell of the spore elongated slightly, and the resting nuclei became somewhat more difficult to stain.

Discussion

It was found in this study that the nuclei of the asci from different perithecia differed in their ability to take up stain. According to McClintock (10), who used different strains of *N. crassa*, the genetics of the fungus strongly influenced the success of the staining procedure. It would appear from the current investigation that the choice of cultured material of the fungus, selected for good stainability, might facilitate future studies of the chromosomes.

Killian (9) has claimed that asci universally arise from croziers. Frey (4) did not see croziers. It has been observed during the course of this study that

hooks were absent in certain perithecia, which contained instead large budding cells which developed into ascuslike structures. Although the origin and further development of these cells were not followed in this study, it appears that both Killian and Frey might be correct and that asci could originate through both processes. Such a dual origin may be common among the Pyronomycetes.

Backus and Keitt (1) suggested a haploid number of four to six chromosomes from observations of the first and second meiotic metaphase. Day *et al.* counted seven chromosomes; their Figs. 3, 8, and 9 show an early anaphase I rather than a late metaphase I and indicate that a few bivalents have already separated. Such a stage is not favorable for counting chromosomes because of the difficulty of distinguishing univalents from bivalents and the consequent risk of overestimating chromosome numbers. In the current study, although chromosome counts could not be made in the vegetative hyphae, the haploid number of seven chromosomes has been repeatedly observed at the metaphase of the crozier and in the prefusion nuclei of the elongating penultimate cell. Furthermore, this same number has been counted during pachytene, diakinesis, metaphase I and II of meiosis, and during the mitotic metaphases in the ascus.

Although it was not the primary purpose of this study to make a chromosome diagram of *V. inaequalis*, an attempt was made to characterize some of the seven chromosomes. It must be understood that descriptive comments are not definitive, but it is suggested that, with additional studies, it may be possible to construct an idiogram of the chromosome complement in this fungus.

Synapsis in *V. inaequalis* is of the precocious type which seems to be a general feature among the higher ascomycetes (5, 11, 14).

It is difficult to determine conclusively from squashed preparations whether or not nuclei or spores are able to pass each other in the ascus. However, the indications are that some nuclei may exchange their position, and that this could take place more easily during the third division than during the second, since at telophase II (Fig. 25) the nonsister nuclei are farther apart than they are in the third telophase. Keitt (6) found that exchange of spore position in the ascus was uncommon.

The first two divisions in the ascus constitute a proper meiosis and bring about the reduction in the number of chromosomes. The third and the fourth divisions are mitotic. This finding is indirect evidence that only one nuclear fusion takes place in the sexual cycle of *V. inaequalis*.

Genetic markers are known for six chromosomes (2, 3). The current study as well as that of Day *et al.* suggests that a seventh linkage group exists.

Acknowledgments

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INFECTION OF BARLEY BY CLAVICEPS PURPUREA[1]

W. P. CAMPBELL[2]

Abstract

The development of infection of the ovary of Newal barley (*Hordeum vulgare* L.) by the ergot fungus, *Claviceps purpurea* (Fr.) Tul., was traced from the time of inoculation until formation of the sclerotia. Penetration took place within 24 hours near the base of the ovule. Initially, colonization was intercellular and confined to the ovary wall but by the fourth or fifth day the fungus began to move intracellularly, penetrating the ovule and also growing out onto the surface of the ovary to form a hymenium. Spores and honeydew were produced within five days of inoculation. The advancing hyphae were seldom found in stylar or stigmatic tissue and then only after the parasite was well established. Usually these structures are pushed upward to form a cap on the maturing sclerotium.

Introduction

Ergot, caused by *Claviceps purpurea* (Fr.) Tul., has long interested man, but it was not until 1711 that Geoffroy first suggested that it was a separate plant, a fungus growing on the ovaries of wheat. This view was confirmed by Münchhausen in 1764 and by several other writers in the latter part of the eighteenth century, finally to be brought forward again by DeCandolle (1815) as a new idea of his own. The above works have been cited by Barger (1) in his book *Ergot and Ergotism.*

It was not, however, until Tulasne (7) in 1853 studied this fungus that anyone seemed to have understood how it invaded, colonized, and replaced the ovaries in the infected florets. Kühn (4) in 1865 confirmed and extended the work of Tulasne and also grew the fungus on artificial medium. Engelke (2) in 1902 and Kirchhoff (3) in 1929 agreed that spores, either conidia or ascospores, land on the stigmata and germinate in the fluid secreted there. Engelke asserted that the fungus penetrates the stigma and grows down the conducting elements to the base of the ovule in much the same manner as does the pollen tube. From here it was supposed that the colonization of the ovary was in an apical direction because it already had been established by Kühn (4) that the sphacelia is always found first at the base of the ovary and grows upward from there. Kühn also stated that a heavy development of sphacelia is present at the base of the ovary while the internal tissues are still intact. Kirchhoff (3) agreed that the spores germinate on the stigma but stated that the fungus grows down over the external surface of the ovary to find a point of ingress at its base. He and Engelke thought that stigmata were of little importance as avenues of penetration, and Kirchhoff stated further that ingress is at the point of attachment of the ovary to the receptacle. From this area, he noted, the sphacelial strands grow upward through the

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tissues of the ovary. Milovidov (5) wrote that, in artificial inoculation of rye ears with conidia, the spores germinated and formed dense mycelium, the hyphae of which attack the pistil and anthers and sometimes other flower parts. The hyphal mass presses on the inner cells of these organs and destroys them, using their contents as food, the debris being forced upward to form a cap on the maturing sclerotium. He also differentiated between sphacelial and sclerotial cells.

An analysis of the literature presents many contradictions in respect to the pathway of infection and the subsequent development of the pathogen in the host. The present work was undertaken to establish the histological pathology of ergot infection from the time of inoculation until maturation of the sclerotia.

Materials and Methods

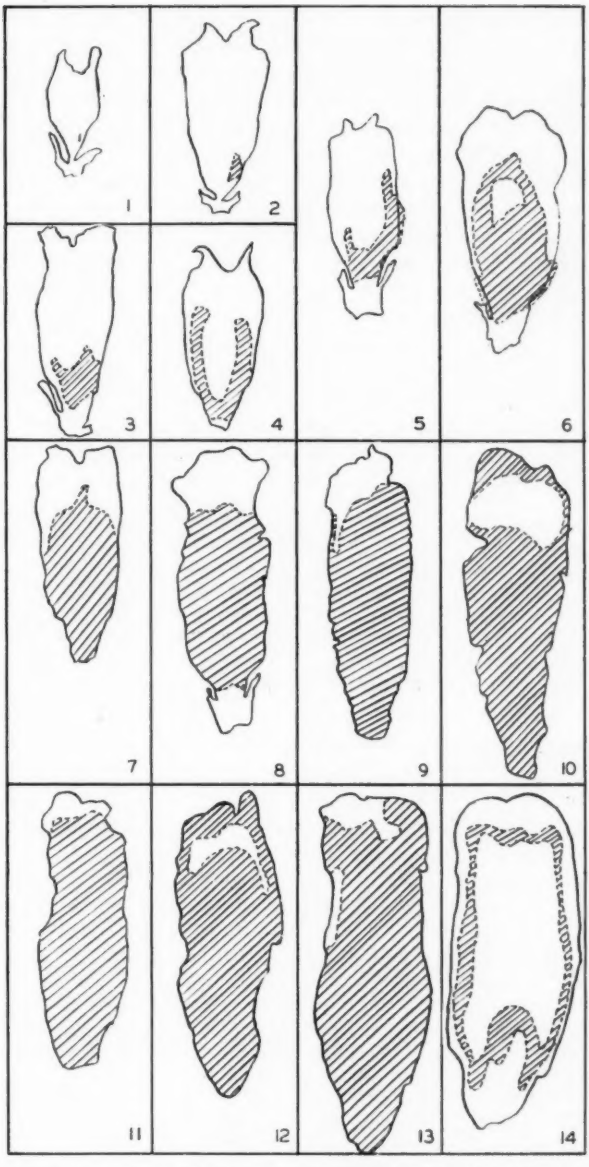
Newal barley grown in the greenhouse was inoculated with a suspension of *C. purpurea* conidia blown into the florets at flowering time with an atomizer. Florets were harvested at 24-hour intervals following inoculation and immediately fixed in a solution of the following formulation: 6 parts of isopropyl alcohol, 3 parts of propionic acid, and 1 part each of petroleum ether, acetone, and dioxane as outlined by Newcomer (6). The florets were then imbedded in paraffin, sectioned with a microtome, and stained by Conant's quadruple stain. The oldest material examined was collected 17 days after inoculation.

Results

Within 24 hours of inoculation the conidia germinated and produced a limited mycelium which penetrated the ovary near its base (Figs. 1 and 15). During the second and third days the mycelium was found passing through the ovary wall intercellularly (Figs. 16 and 17) until the integuments were reached (Fig. 18). While the integuments appeared to form a temporary barrier the fungus grew well in a generally upward direction through the ovary wall to entirely surround the ovule. By the fourth or fifth day the mycelium developed intracellularly as well as intercellularly (Figs. 19 and 20). At about this time hyphae began to erupt on the surface of the ovary (Fig. 21), there to produce the first conidia (Fig. 22), and the resistance of the integuments broke down to allow penetration and colonization of the ovule (Figs. 23 and 24). Cavities which became lined with a hymenial layer of conidiophores began to appear throughout the mycelial mass.

The fungus has not at any time been found to penetrate the style and stigma until at least a week after initial infection and then rarely. This penetration has been found only after these flower parts have already been overgrown by sphacelia from below.

The host tissue is not completely destroyed. There is usually a cap of uninvaded material from the stylar region on the top of the sclerotium or there may be islands of intact host tissue scattered throughout the ergot body.



FIGS. 1–14. Line drawings showing the progressive colonization of barley ovaries by *Claviceps purpurea*. Shading indicates area infected. FIG. 1. Ovary showing penetration near its base 24 hours after inoculation. FIGS. 2–13. Ovaries 2, 3, 4, 5, 6, 8, 9, 10, 12, 14, 15, and 17 days after inoculation, respectively. FIG. 14. An intact-appearing ovary which did not produce honeydew but which had a darkened, water-soaked appearance.

As the sclerotium begins to develop, starting always from the base, the conidial production ceases and the cavities within the fungus mass become filled with mycelium. There are two types of cells present in the mature sclerotium: relatively fine hyphal cells which penetrated and ramified through the ovary of the host; and large-diameter, irregular, barrel-like cells of later growth.

It has been noted that under certain circumstances, possibly late infection, the host plant produces seeds which seem normal. On sectioning, however, it is found that some of these contain a layer that appears to be intact but which has an irregular layer of *Claviceps* mycelium under it surrounding the endosperm (Fig. 14). An intact embryo is yet to be found in one of these seeds.

Downward development of the fungus from the infection court is limited, and it does not appear to penetrate very far into the receptacle. There is a rather definite line of demarcation between colonized and uncolonized host cells in the receptacle.

Figures 1 to 13 constitute a diagrammatic summary of the colonization of an ovary of barley and the development of a sclerotium.

Discussion

It has not been possible in this study to corroborate fully the findings of any one earlier author. The different stages of the infection phenomenon follow one another very rapidly. Unless observations are initiated within one day after inoculation and continued daily thereafter the various events may well be missed or misinterpreted. Failure to adhere to this procedure is probably the main reason for the apparent discrepencies of interpretation in the literature. If one were to start examining material after the production of honeydew had commenced it would be very easy to find support for the views of any or all previous workers. By that time the ovary was colonized and overrun by the fungus and even the stigma and style were occasionally found to be colonized, but this was only as a consequence of invasion from tissue below them.

Often we found sclerotia with a well-developed remnant of the seed on its apex, but we never found a seed with ergot tissue on its apex. Therefore it seems apparent that invasion and colonization of the ovary by the fungus

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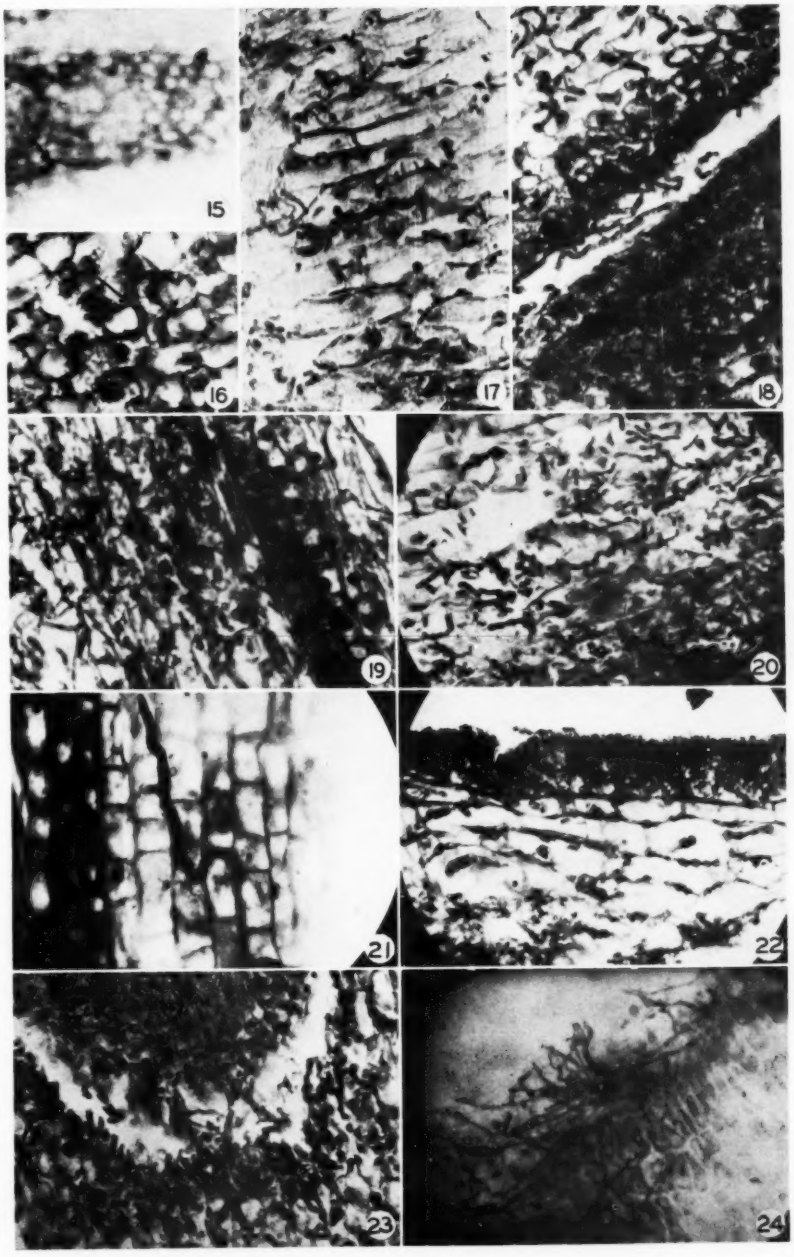
FIG. 15. Initial penetration 24 hours after inoculation. (×500)
FIG. 16. Intercellular colonization 2 days after inoculation. (×1200)
FIG. 17. Intercellular colonization 3 days after inoculation. (×700)
FIGS. 18 and 19. Advance of the mycelium temporarily halted at the integuments. (×700)
FIG. 20. Intercellular and intracellular colonization with attendant breakdown of the host tissue begins about the fourth day. (×700)
FIG. 21. Emergence of mycelium initiates the development of a fruiting layer on the surface of the ovary. (×1200)
FIG. 22. Well-developed fruiting layer on outer surface of ovary 5 days after inoculation. (×700)
FIGS. 23 and 24. Invasion of the ovule. Fifth and sixth days. (×700)



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must be from the bottom upward. The results of the present work, along with those of Kühn (4) and Kirchhoff (3), confirm this view. Unlike Kirchhoff, however, the author has not been able to find that spores germinate on the stigma and grow down over the surface of the ovary. Limited mycelial development was found on the exterior of the ovary and penetration appeared to be between the cells.

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INHIBITION OF RESPIRATION, GERMINATION, AND GROWTH BY SUBSTANCES ARISING DURING THE DECOMPOSITION OF CERTAIN PLANT RESIDUES IN THE SOIL[1]

Z. A. PATRICK AND L. W. KOCH[2]

Abstract

Substances capable of markedly inhibiting the respiration, germination, and growth of tobacco seedlings were obtained after residues from timothy, corn, rye, or tobacco plants had been allowed to decompose under appropriate conditions in the soil. Bio-assay tests, based on manometric methods, made it possible to determine some of the conditions under which the decomposition gave rise to phytotoxic by-products and to obtain rapidly an accurate measure of the degree of their toxicity. Among the factors influencing the production of the toxic products, the species and stage of maturity of plant material added, the water content and pH of the soil, and length of decomposition period seemed most important. Substances capable of reducing the respiration of tobacco seedlings by 50 to 90% were consistently obtained when any of the above-mentioned plant residues had been decomposing for 15 to 25 days under conditions of high soil moisture and when the pH of the soil solution was below 5.5 throughout this period. Timothy gave rise to substances possessing the highest toxic activity, followed by corn, then rye, and finally tobacco. Aqueous extracts of unamended soil or of macerated plant tissues prepared either before decomposition had taken place or when decomposition was inhibited by autoclaving the soil were not toxic.

The toxic substances, obtained by water extraction, exhibited an inhibiting effect on respiration of tobacco seedlings after an exposure of less than one hour and also induced darkening and necrosis of root cells. Some extracts affected the cells of the apical meristem most severely while others affected only the cells of the elongation region.

While the toxic substances have not been identified, they were relatively nonspecific in their action, affecting tobacco, timothy, and barley in approximately the same manner and in many instances possessed also antifungal activity. They were soluble in water, insoluble in petroleum ether, stable in acid, and most active in the pH range 4.5 to 5.8. They were precipitated by alkali and the activity was markedly reduced when readjusted to the acid range. They were also heat stable and did not lose their activity in storage at 1°–3° C. provided they were covered by a layer of toluene.

Because of their rapid production and the marked injurious effects exerted on various plants it is believed that these toxins may perform a significant role in the field as the primary cause of some root rots and in predisposing plants to attack by organisms not normally regarded as pathogenic.

Introduction

The return of plant residues to the soil has long been recognized as being of agronomic importance especially as related to tilth, and maintenance of organic matter. Such practice, however, sometimes results in deleterious effects on succeeding crops. The precise mechanisms of the injurious effects on plants are not clearly understood. Some investigators attribute the harmful effects of crop residues to the depletion of nutrients or immobilization of nitrogen by soil organisms during the decomposition processes (20, 25). Others have suggested that the harmful effects were mainly due to their influence on the microbiological balance with the resulting effect on the survival and virulence of

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many soil pathogens (13, 21, 26). These and other points of view have received much attention over the years and yet much of the information on this subject, which is considered to be a phase of the general problem of soil toxicity, is still contradictory. Thus the actual existence and presumed importance of such naturally occurring soil toxins are still matters of controversy and, although many of the earlier investigators were able to isolate and identify substances toxic to plant growth from certain soils (28), most of their findings were of an indirect nature and the origin of these substances was not definitely established. Relevant literature on the general subject of soil toxicity has recently been comprehensively reviewed by Bonner (8) and Audus (2).

More recent evidence has been more conclusive (4, 9, 14, 16) and there appears to be little doubt that substances toxic to living plants can originate from excretions of roots and underground stems of certain living plants (5, 6, 19, 22), or may be liberated from plant residues during their decomposition (3, 4, 11, 23). Frequently it may not be possible to distinguish between these two sources. However, many of the investigators concerned with crop sequence and the so-called soil sickness effects have generally come to believe that the residues of certain plant species are the most important contributors to soils of compounds toxic to plants.

Although many of the facts concerning soil toxicity, its existence, nature, origin, and function, still remain contentious, it is generally agreed that the incorporation and subsequent decomposition of plant residues in the soil can have important implications not only in the field of horticulture but also to plant pathologists. In this respect also there is an increasing volume of evidence to suggest that many soil toxins arising from plant residues may predispose or condition the plant to invasion by many root-rot-producing organisms (10, 24).

In view of the growing recognition that soil toxins may be playing an important role in the etiology of many root rots (10, 13, 17, 24), as well as their probable importance in connection with certain root disorders of tobacco in Ontario which at present cannot be assigned completely to parasitic organisms, this study was undertaken. The main purpose of the work reported in this paper was to determine whether and under what conditions decomposition of the residues of some of the crops grown in this area would result in the production of substances toxic to plants and to determine the various morphological and physiological changes that such substances induce in the plant in the absence of other causal factors which could modify its susceptibility to attack by some of the root-rot-producing organisms.

Materials and Methods

Crop Residues and Soils Used

Materials from timothy, rye, tobacco, and corn plants were used in the decomposition studies. The soil and plant materials for most of the experiments were obtained from the Science Service field plots in which the above-mentioned four crops had been grown for at least 2 consecutive years. In the

controls the soil used was from the same series of plots except that the plots had been fallow and kept free of plant growth. Soils from the Delhi and other tobacco-growing areas were also used in many of the experiments.

The plant materials which were added to the various soils were obtained at three different stages of growth: young, intermediate, and nearly mature. In the young stage, about 5 to 6 weeks after planting, the plants were 12 to 18 in. high and succulent. In the intermediate stage, 6 to 8 weeks after planting, the plants were 20 to 36 in. high and were close to the flowering stage. At near maturity, 10 to 14 weeks after planting, the corn and tobacco plants were still quite green while the rye and timothy were only partially green in color. Entire plants including the roots were harvested at the stages of growth mentioned, cleansed of soil, cut up into pieces approximately 1 in. long, and were either used immediately or air-dried and stored for future use.

The soil was obtained from the same plots which grew each of the above crops. Soil samples were taken to a depth of 8 in., from four to six locations within each plot. The plants and roots in this layer were separated and the soil made into one composite sample for each plot. The soil throughout all the plots appeared to be a uniform Fox sandy loam with a pH range of 6.2 to 6.8. All plots had received identical fertilizer applications.

Decomposition and Extraction Procedures

To 1000-g. quantities of fresh soil were added 250 g. fresh weight, or 60 g. air-dry weight, of the various plant materials including roots, stems, and leaves; the materials were mixed thoroughly and placed in 1-gal. sterile glazed crocks. Each of the plant materials was added to the same soil in which it had been growing and no plant materials were added to the fallow soil. Water was then added to each of the crocks. In some instances enough water was added to saturate the contents while in others the moisture content was adjusted to approximately field capacity. The term field capacity is used throughout these tests to denote that amount of moisture which is retained in the soil after gravitational drainage of excess water has taken place. This condition was attained by adding water to the soil and allowing the excess to drain away. The crocks were covered with aluminum foil to reduce water loss during the decomposition period and were kept at 60° to 70° F. for periods varying from 0 to 30 days. For each of the series enough crocks were prepared to allow extraction from the contents of two crocks after decomposition periods of 0, 5, 10, 15, 20, 25, and 30 days for each of the crops used. Parallel series of crocks were prepared every 30 days and, over a period of 3 years, 18 such decomposition series were carried out. In another series of tests the soil and plant materials were placed in 2000-ml. Erlenmeyer flasks which were plugged with cotton and autoclaved for 40 minutes at 18 lb. pressure before being allowed to incubate for the various periods.

After each incubation period two crocks of each crop were removed and 1000 ml. of water added; the contents were stirred for 15 to 20 minutes and allowed to settle. The liquid was then decanted and its pH determined.

After being centrifuged at approximately 4500 r.p.m. for 30 minutes, the liquid was then filtered first through an 8-lb., and again through a 16-lb., Seitz filter to remove soil particles, microorganisms, and undecomposed plant debris. The precipitate and remainder of the crock contents were discarded. The filtrates were covered with a layer of toluene, placed in cold storage, and kept at 1–3° C. until used. The various filtrates were tested for phytotoxic properties usually within 5 days after preparation.

Tests for Toxicity

A method, similar to that used to demonstrate the toxic nature of breakdown products from peach-root residues (23), was used in this study as the principal assay method to determine whether the above-mentioned filtrates, containing decomposition by-products of the various plant residues, possessed phytotoxic properties and also to evaluate the relative toxicity of such substances. In this method, which is based on manometric techniques, the respiration rates of tobacco seedlings, previously exposed to aqueous extracts containing the decomposition products of the various plant residues, are compared with respiration rates of similar seedlings exposed to aqueous extracts of soils which do not contain such residues or with respiration rates of tobacco seedlings exposed to 0.01 *M* phosphate buffer solution.

The effect of each filtrate on the respiration of tobacco seedlings was tested as follows. Tobacco seed of the variety Harrow Velvet was surface sterilized and placed in Petri dishes containing 1.2% sterile agar. The plates were then placed in an incubator held at 25° C. The seedlings were used 6 days after being sown, at which time they had germinated and grown approximately 6 mm. At this time sterile and apparently uniform tobacco seedlings from at least four Petri dishes were collected and placed in two other dishes containing sterile distilled water. Twenty milliliter portions of each filtrate to be tested (previously adjusted to pH 5.3–5.8 with HCl or NaOH) were placed in sterile Petri dishes, two separate Petri dishes for each filtrate. Into each of the dishes containing the filtrates, 100 of the 6-day-old tobacco seedlings were then placed. The seedlings were then allowed to remain overnight (16 hours) in the various filtrates at room temperature, which was maintained at approximately 20° C. The same number of tobacco seedlings was also placed in dishes containing sterile 0.01 *M* phosphate buffer solution (pH 5.3–5.8) and allowed to remain there for the same period. The next day the tobacco seedlings were removed from each of the filtrates, rinsed in sterile distilled water, and placed in Warburg flasks containing 2.2 ml. of sterile 0.01 *M* phosphate buffer solution. Fifty seedlings were placed in each flask; thus, there were in each instance four flasks with seedlings which had been pre-exposed to the same filtrate. The oxygen uptake of the tobacco seedlings pre-exposed to the various filtrates was then determined in the conventional manner by the direct method of Warburg (30). Filter paper immersed in 0.2 ml. of 20% KOH placed in the center well was used to absorb the CO_2 produced. The oxygen uptake of tobacco seedlings pre-exposed to the phosphate buffer solution was used as the control and given the respiration value of 100%. The

respiration of the tobacco seedlings pre-exposed to the various filtrates was then calculated as percentage of the control. All tests were carried out at 20° C. and readings of the oxygen uptake were taken every hour for 6 hours. Each filtrate was tested on at least three separate occasions. Whenever differences in the O_2 uptake of flasks containing identical filtrates exceeded 15% the tests were repeated.

Additional bio-assay tests were carried out in which the effect of the various filtrates on germination and growth of tobacco, barley, and timothy seed and seedlings was also determined. The test procedure was similar for all three types of seed and only the test with tobacco will be described. Two layers of filter paper were placed in the bottoms of Petri dishes, the walls of which were then lined with absorbent cotton and then these dishes were autoclaved for 15 minutes at 18 lb. pressure. Each of the filtrates to be tested was then apportioned to the dishes in 25 ml. amounts. After the liquid was absorbed by the filter papers and the cotton liner, approximately 100 Harrow Velvet tobacco seeds were sprinkled on the filter paper impregnated with the various filtrates and incubated at room temperature. Results were obtained after 7 days of incubation. In the controls, phosphate buffer solution was added in place of the various filtrates. After the 7-day incubation period 25 seedlings, selected at random, were removed from each dish, placed on glass slides along with a millimeter scale, and photographed with the camera locked in position. After printing, measurements were made of each seedling (see Figs. 11 to 13) and the relative toxicity of the various filtrates was evaluated from this data.

Modifications of the above procedures and additional methods are described below in conjunction with the experiments in question.

Observations and Results

Decomposition of Various Plant Residues in the Soil and the Effect of the Resulting Products on Respiration of Tobacco Seedlings

Effect of Soil Moisture During the Decomposition Period on Relative Toxicity of the Resulting Products

Various investigators have reported that soil toxicity due to organic constituents is mainly associated with soils characterized by poor aeration or excessive moisture conditions (21, 24, 28). Many of the specific organic compounds detrimental to plant growth also have been isolated most frequently from such soils (29). From such results it has been generally concluded that most of the toxic compounds occurring in such soils are formed chiefly during the decomposition of plant residues and other soil organic matter under conditions of high soil moisture. It has not been shown, however, that the substances arising during decomposition of plant residues under conditions of high soil moisture are more toxic to plants than those formed during decomposition under normal moisture conditions or that the concentrations of such substances are different under these conditions. Experiments were therefore carried out

to determine whether the decomposition of similar plant residues, in essentially identical soils but under different moisture conditions, would give rise to substances which would exert different effects on the respiration of tobacco seedlings. In the first series of tests the various plant materials were allowed to decompose in soils held at two moisture levels, saturation and field capacity. For the tests, two series of crocks were prepared simultaneously, soil and plant materials from the same composite samples being used. To one series of crocks sufficient water was added to saturate the contents, while in the other the moisture level was adjusted to field capacity by allowing the excess water to drain away. Both series were then kept at 60°–70° F. for 20 days. At that time water extracts were obtained from each of the crocks and the effect of the substances formed during decomposition under the two conditions on the respiration of tobacco seedlings was determined in the manner described earlier. The results are presented in Table I.

TABLE I

OXYGEN UPTAKE OF TOBACCO SEEDLINGS AS AFFECTED BY THE PRODUCTS OF DECOMPOSITION OF PLANT RESIDUES IN SOILS HELD AT SATURATION AND AT FIELD CAPACITY

Plant material added to soil	After 20-day decomposition period at:			
	Saturation		Field capacity	
	pH range*	O_2 uptake†	pH range	O_2 uptake
Soil only	6.4–6.6	128	6.4–6.6	131
Timothy	4.8–5.2	18	5.9–6.4	90
Rye	5.3–5.8	45	6.0–6.6	104
Corn	4.9–5.4	28	6.0–6.5	99
Tobacco	5.5–5.9	68	6.9–7.5	112
Control 125 (±8)‡				

*pH range after the 20-day decomposition period; pH of each extract was adjusted to 5.3 prior to testing.

†O_2 uptake, in µl. of O_2 after 6 hours at 20° C., of 50 Harrow Velvet tobacco seedlings. In each instance the tobacco seedlings were exposed for 16 hours to the various extracts then returned to phosphate buffer (pH 5.3) before their O_2 uptake was determined. Each figure is based on the average of four different decomposition series for each of which three determinations of four replicates each were made.

‡O_2 uptake, in µl. of O_2 (after 6 hours at 20° C.), of comparable seedlings pre-exposed (16 hours) to 0.01 *M* phosphate buffer solution; based on the average of 12 determinations.

From the results in Table I it will be noted that, when the two above-mentioned soil moisture levels were maintained during the decomposition processes, substances were formed which induced different effects on the respiration of tobacco seedlings. Whereas the substances formed when decomposition of plant residues took place in soils under saturation conditions strongly inhibited the oxygen uptake of tobacco seedlings, those formed in soils held at the lower moisture levels had relatively mild inhibiting effects on the oxygen uptake of the tobacco seedlings. Such differences, however, could have been partly due to differences in concentration since the toxic products were not totally eliminated even when the decomposition occurred in soils held at the lower moisture levels inasmuch as the products arising from

the decomposition of the plant residues, especially those from timothy or corn, were still quite toxic. Extracts of soils to which no plant residues had been added, maintained at either of these two soil moisture levels, had no inhibiting effects on the respiration of tobacco seedlings and presumably did not contain toxic substances.

Additional experiments were carried out in which the two soil moisture levels mentioned above were varied during the test period. Decomposition was carried out on the one hand for 15 to 20 days in soils held at field capacity followed by saturation and, on the other, for the same period under saturation conditions followed by a dry period. In general, it was noted that, if decomposition was carried out for 15 to 20 days under moisture levels equal to field capacity followed by flooding, the resulting extracts, obtained 3 to 5 days following the flooding, were highly toxic. On the other hand, if the decomposition was carried out under saturation conditions for the same period then adjusted and maintained at field-capacity moisture levels, the toxicity decreased slowly and was reduced by only one half 10 days after the return to the drier soil conditions.

Effect of Plant Residue and Decomposition Period on Relative Toxicity

Experiments were next carried out to determine the relationship between the type of plant material added to the soil, the decomposition period, and the relative toxicity of the resulting products. In these experiments all plant materials used were at the intermediate stage of growth and enough water was added to saturate the contents of the crocks. Procedures with respect to the preparation of plant materials, decomposition, extraction, and assay were similar to those described earlier.

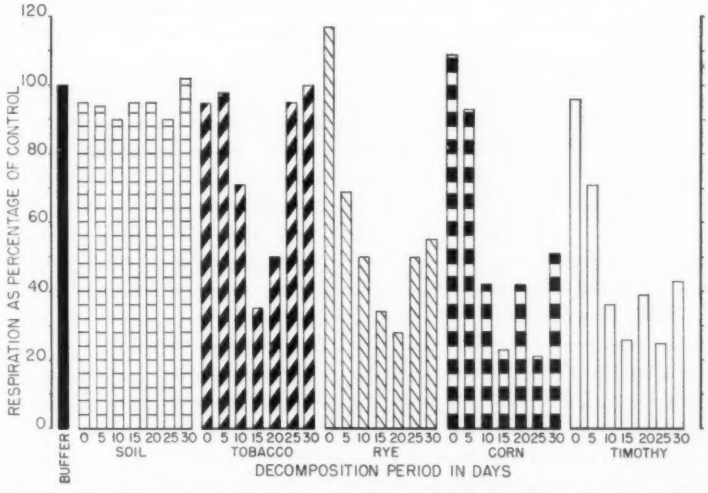

FIG. 1. Relative toxicity, based on percentage inhibition of respiration of tobacco seedlings, as affected by type of plant material added to the soil and period of decomposition.

The results of these experiments are presented in Fig. 1. In these tests the oxygen uptake of tobacco seedlings after they had been exposed for 16 hours to the various extracts was compared with the control, i.e., similar seedlings exposed to 0.01 *M* phosphate buffer solution for the same period of time. The results, calculated as percentages of the control, are based on average results from nine different decomposition series carried out over a 3-year period and involving more than 400 extracts from the various crops.

From the data summarized in Fig. 1 it will be noted that substances which adversely affect the respiration of tobacco seedlings arise during only certain stages of decomposition of the various plant residues. Moreover extracts from fallow soil to which no plant residues had been added had little or no effect on the respiration of tobacco seedlings as was evidenced by the fact that the respiration of seedlings which had been exposed to aqueous extracts from such soils was essentially identical to that of seedlings which had been in the buffer solution for the same period of time. On the other hand, the respiration of seedlings exposed to extracts of soil to which various plant residues had been added varied and depended on the type of residue added to the soil and the length of the decomposition period. When the plant material was added to the soil and extracted within a few hours of preparation, most of the extracts only slightly affected the respiration of tobacco seedlings. Thus, the extracts from tobacco and timothy produced a slight inhibiting effect, while rye and corn produced some stimulation of respiration. After 5 days of decomposition, differences began to appear; extracts containing decomposition products of tobacco or corn still had little effect on respiration whereas extracts of soil in which rye or timothy had been decomposing induced about 30% inhibition of respiration. After the 10-day period, extracts of soil in which timothy, corn, or rye had been decomposing contained substances which had marked inhibiting effects on respiration of tobacco seedlings and extracts of soil in which tobacco had been decomposing also began to exert inhibiting effects at this time. By the time the various plant residues had been decomposing in the soil for 15 days, all extracts, except that of fallow soil, contained substances which were highly toxic and the respiration of tobacco seedlings was markedly reduced when exposed to any of the extracts. After the 20-day decomposition period the toxicity of the extracts still remained high and respiration, in most cases, was inhibited by more than 50%. After the 25-day period, the toxicity of extracts containing decomposition products of tobacco had almost disappeared and the toxicity of rye was somewhat reduced. Extracts of soil in which corn and timothy had been decomposing, however, were still highly toxic. A further reduction in the relative toxicity of most of the extracts took place by the 30th day, although the rate of its reduction was not the same for the various plant materials used. Thus, while extracts of soil in which tobacco had been decomposing for 30 days no longer inhibited the respiration of tobacco seedlings, those containing decomposition products of timothy or corn still inhibited the seedling respiration by about 50%.

Effect of Plant Maturity on Relative Toxicity and Duration of the Toxic Period

In view of the marked changes in the chemical composition of plants during the various stages of growth (15, 20) experiments were next carried out to determine the effect of maturity on degree of toxicity of the decomposition products and relative rate of their production. The various plant materials were added to the soil at three different stages of their development, namely young, intermediate, and mature. Decomposition, as in the previous experiments, was carried out under high soil moisture conditions and the relative toxicity of the resulting products was again based on percentage inhibition of respiration of tobacco seedlings.

The results are summarized in Table II, in which, for ease of comparison, the extracts are divided into four activity categories, nontoxic, mild, intermediate and highly toxic, on the basis of percentage inhibition of respiration they induced. From these results it will be noted that the stage of maturity at which the plant residues were added to the soil was an important factor in determining the time required for toxic substances to be formed, as well as in determining the relative toxicity of these substances. When residues from young plants are added to the soil, substances which inhibit the oxygen uptake of tobacco seedlings are produced during relatively early stages of the decomposition process. Most of these substances exert relatively mild effects on respiration of tobacco seedlings and are inactivated quite rapidly during the subsequent stages of the decomposition. Under laboratory conditions most of the toxic effects disappear by the 25th day of decomposition. When residues

TABLE II

EFFECT OF PLANT MATURITY AND PERIOD OF DECOMPOSITION ON RELATIVE TOXICITY OF THE RESULTING PRODUCTS

Age of plant material added to soil	Type of plant	Decomposition period (days) at 60–70° F.						
		0	5	10	15	20	25	30
Young (5 to 6 weeks after planting)	Soil only	−*	−	−	−	−	−	−
	Timothy	−	+*	++	+++	++	−	−
	Rye	−	−	+	++	+	−	−
	Corn	−	−	+	++	+	−	−
	Tobacco	−	−	+	+	−	−	−
Intermediate (6 to 8 weeks after planting)	Soil only	−	−	−	−	−	−	−
	Timothy	−	+	+++	+++	++	+++	++
	Rye	−	+	++	+++	+++	++	++
	Corn	−	−	++	+++	++	+++	++
	Tobacco	−	−	+	+++	++	−	−
Mature (10 to 14 weeks after planting)	Soil only	−	−	−	−	−	−	−
	Timothy	−	−	−	++	++	+++	+++
	Rye	−	−	−	+	++	+++	++
	Corn	−	−	−	+	++	+++	+++
	Tobacco	−	−	−	+	++	++	−

*Toxicity rating: − =nontoxic: extracts which inhibit respiration of 6-day-old tobacco seedlings by less than 19%; +, ++, +++=mild, intermediate, and highly toxic, producing inhibition of respiration of 20–40%; 41–60%, and 61–95% respectively.

from plants at the intermediate stage of growth are incorporated into the soil, toxic substances are formed somewhat later in the decomposition cycle but they are more toxic and persist for a longer period of time. When residues from mature plants are added to the soil, toxic products do not arise until during the relatively late stages of decomposition.

Effect of Type of Plant Residue and Decomposition Period on Soil pH and Relative Toxicity of the Resulting Products

Although acidity is known to play an important role in soil toxicity, the relation of acidity to the formation of toxic decomposition products is still not clearly understood. Thus certain plant residues when added to the soil increase soil acidity during their decomposition while others produce very little change (20, 25) and it has been noted that the lowest yield of the succeeding crop was obtained following those plant residues which created the highest acidity. The harmful effects to succeeding plants were considered by some investigators to be due mainly to the unfavorable influence of such plants on the soil reaction (20, 25), while others believed that the harmful effects were mostly due to toxic organic compounds which were produced during the decomposition of plant residues under conditions of high acidity (20, 21). Experiments were therefore undertaken to determine whether some of these questions could be clarified using inhibition of respiration as the index of toxicity. The purpose of the first series of tests was to determine the relationship between the degree of toxicity of the various extracts and their pH. For these tests the soil and plant materials were obtained from field rotation plots mentioned above. The initial pH of the soil ranged between 6.2 and 6.8. At the end of each 5-day period pH of the soil solutions in which the various plant residues had been decomposing was determined. The relative toxicity of the extracts which were obtained at the same time were also determined by means of the tobacco seedling respiration test. The results are summarized in Table III, from which it is evident that, following the addition of the various residues to the soil samples, the pH of the soil or of resulting extracts was not altered appreciably as long as no decomposition had taken place. Likewise the extracts obtained at this zero-day decomposition period exerted no inhibiting effects on the respiration of tobacco seedlings, which in many instances was actually stimulated. As the decomposition period was extended, the pH of the extracts changed considerably, the pH of some extracts having shifted from the 6.2–6.9 range to the 4.8–5.9 range after only a 5-day decomposition period. Corresponding with the change in pH, there was also a change in the relative toxicity of the extracts, inhibition of respiration of tobacco seedlings being obtained only with extracts whose pH was below 6.0 at this time. As the decomposition progressed, more and more extracts were obtained with pH in the 4.8 to 5.9 range. Although decomposition of the same types of plant materials for equal periods of time did not in all instances give rise to substances having the same inhibiting effects on the respiration of tobacco seedlings, there was a high degree of consistency between the relative toxicity of extracts and their pH. Thus, for example, extracts with pH of 4.8 to 5.3 were

TABLE III

EFFECT OF PLANT SPECIES AND DECOMPOSITION PERIOD ON pH AND RELATIVE TOXICITY OF THE RESULTING SOIL EXTRACTS

Decomposition period (days) at 60–70° F.	pH range*	Type of plant material added to the soil: Soil only — No. of extracts†	Soil only — Toxicity rating‡	Timothy — No. of extracts	Timothy — Toxicity rating	Rye — No. of extracts	Rye — Toxicity rating	Corn — No. of extracts	Corn — Toxicity rating	Tobacco — No. of extracts	Tobacco — Toxicity rating
0	6.2–6.5	20	–	29	–	22	–(s)	24	–(s)	8	–
	6.6–6.9	16	–	7	–	14	–(s)	12	–(s)	16	–
	7.0–7.3	0		0		0		0		12	–(s)
5	4.8–5.3	0		8	++	4	++	2	+	2	+
	5.4–5.9	0		16	+	19	+	6	+	1	+
	6.0–7.0	36	–	12	–	13	–	28	–	33	–
10	4.8–5.3	0		14	++	2	++	10	++	1	++
	5.4–5.9	0		16	++	23	+	17	++	1	+
	6.0–7.0	36	–	6	–	11	–	9	–	34	+
15	4.8–5.3	0		16	+++	10	++	9	+++	6	+++
	5.4–5.9	0		13	++	15	++	21	++	7	++
	6.0–7.0	36	–	7	–	11	–	6	–	23	+
20	4.8–5.3	0		12	+++	13	+++	7	++	3	++
	5.4–5.9	0		16	+++	14	++	20	++	4	++
	6.0–7.0	36	–	8	–(+)	9	–(+)(s)	9	–(+)(s)	29	+
25	4.8–5.3	0		7	+++	4	+++	9	+++	0	
	5.4–5.9	0		18	++	20	++	20	++	2	++
	6.0–7.5	36	–	11	–(+)(s)	12	–(+)(s)	7	–(+)(s)	34	–(s)
30	4.8–5.3	0		4	+++	2	+++	3	++	0	
	5.4–5.9	0		18	++	16	++	18	++	2	+
	6.0–7.5	36	–	14	–(+)(s)	18	–(+)(s)	15	–(+)(s)	34	–(s)

*pH range of the aqueous extracts obtained after the various plant residues (at the intermediate stages of growth) had been decomposing in the soil for the periods indicated. The pH measurements were made with a Beckman model G pH meter.

†A total of 36 extracts of each plant material and at each of the decomposition periods was tested. These were obtained from nine decomposition series replicated four times.

‡Toxicity rating was based on percentage inhibition of respiration of 6-day-old Harrow Velvet tobacco seedlings as compared with the buffer control: – =nontoxic: extracts which inhibit respiration of tobacco seedlings by less than 19%; +, ++, +++ =increasing orders of toxicity, producing inhibition of respiration of 20–40%, 41–60%, 61–95% respectively. (+) indicates instances where some of the extracts gave no inhibition of respiration while others in the same series gave inhibition values of 21–30%. (s) indicates cases where some of the extracts also produced stimulation of respiration.

all highly toxic and gave inhibition of respiration values of 65% or greater. Extracts in the pH 5.4 to 5.9 range were generally less toxic than those just mentioned although they still produced marked inhibiting effects on the respiration of tobacco seedlings. The relative toxicities of the extracts in this pH range were more variable, however, and other factors such as decomposition period and the type of plant residue which was added to the soil exerted some influence. For example, most of the extracts which were obtained at the 15- to 20-day decomposition period were highly toxic, while extracts obtained at an earlier or later period were moderately to weakly toxic. Throughout these tests most of the extracts with pH 6.0 or higher produced very little or no inhibiting effects on the respiration of tobacco seedlings, with one notable exception of tobacco which had been decomposing for 15 to 20 days. In this instance inhibiting effects on the respiration of tobacco seedlings was often produced by extracts with pH above 7.0, but toxicity was relatively low and respiration was inhibited by less than 40%. After the 15- to 20-day period the number of extracts in the highly toxic and acid range gradually decreased and by the 30th day more than half of the extracts were not toxic and had a pH above 6.0. These results are especially significant in view of the fact that the pH of soils to which no plant residues had been added did not vary from the initial range of 6.2 to 6.8 throughout the entire test period of 30 days and aqueous extracts obtained from these soils had no inhibiting effects on the respiration of tobacco seedlings.

Because of the consistent relationship between pH and toxicity, from the pH relatively accurate predictions could be made of the degree of toxicity resulting from the decomposition of a given plant residue in the soil. If, for instance, the pH of the soil after a certain decomposition period was below 6.0, it was highly probable that extracts of that soil would be toxic, whereas if it was above 6.0, the extracts were generally either weakly or nontoxic. Once attained, the toxin-producing capacity of each soil could be maintained almost indefinitely at a high level by the addition of plant residue and extracting at 3- to 5-day intervals.

The next series of tests was carried out to determine whether the inhibiting effects on the respiration of tobacco seedlings were due solely to the acidity of the extracts or to the presence in the extracts of toxic decomposition products. In these experiments, to keep them within manageable limits, comparisons were made only between extracts which preliminary tests indicated were either highly toxic or nontoxic. For this purpose extracts which in the other tests had reduced the oxygen uptake of tobacco seedlings by more than 80% were combined and provided the highly toxic solutions used in these and subsequent tests. Other extracts obtained from decomposition of similar plant residues but which did not inhibit respiration of tobacco seedlings formed the nontoxic solutions used in these and subsequent tests.

For this series of experiments four different test solutions were used, namely, 0.01 *M* phosphate buffer, fallow soil containing no decomposing plant residues, the highly toxic, and the nontoxic solutions described above. The pH of

each of the above test solutions was adjusted to pH values of 4.6 to 7.6 with HCl or NaOH. The effect of pH on the relative toxicity of the various solutions was determined in the usual manner. In Table IV there are summarized the results of an experiment in which the decomposition products of timothy were used as the sources of the toxic and the nontoxic test solutions. Similar results were also obtained when decomposition products of other crops were used as the test solutions.

TABLE IV

OXYGEN UPTAKE OF TOBACCO SEEDLINGS IN TOXIC AND NONTOXIC SOLUTIONS AS AFFECTED BY pH

pH	Type of solution			
	Buffer	Soil only	Toxic extract	Nontoxic extract
4.6	92*	94	8	90
4.9	110	106	8	104
5.2	124	120	6	136
5.5	122	118	10	134
5.8	122	120	7	138
6.1	126	127	10	140
6.4	129	128	41	128
6.7	125	122	68	135
7.0	122	126	75	138
7.3	127	129	81	142
7.6	133	135	98	146

*Oxygen uptake, in μl. of O_2, of 50 Harrow Velvet tobacco seedlings after 6 hours at 20° C. Each figure is an average of six determinations of four replicates each.

From the results in Table IV, it is evident that the inhibiting effects of the toxic solutions were not due to acidity, since varying the pH of the buffer solution or the other nontoxic solutions did not have an appreciable effect on the oxygen uptake of the tobacco seedlings. The wide range of applicability of the bio-assay test used in these studies was also evident and it was a relatively simple matter to evaluate accurately the toxic effects which were due solely to the acidity of the solutions and those which were due to the toxic decomposition products formed under acid conditions. Thus, it is clear that, although the toxic substances dealt with in this study are mainly produced under acid conditions, once they are formed they maintain their activity throughout the pH range of 4.6 to 6.1. At pH 6.1 and above, the toxic effects of these substances are gradually reduced as shown by the increase in the oxygen uptake of the tobacco seedlings. The toxic substances apparently were not completely destroyed, however, up to pH 7.6. In similar tests carried out with the nontoxic solutions change of pH from 7.6 to 5.2 did not induce toxicity. In most instances the oxygen uptake of the tobacco seedlings in such solutions was stimulated. As was expected extracts of soil which did not contain decomposing plant residues had no inhibiting effects on the respiration of tobacco seedlings and the oxygen uptake of tobacco seedlings

exposed to such extracts was inhibited only slightly at pH 4.6 to 4.9. This same degree of inhibition was also obtained in the buffer and the nontoxic solutions.

A few additional tests were carried out to determine whether the toxin-producing capacity and the degree of toxicity of a given soil could be altered by adjustment of the soil reaction. For this purpose a number of crocks were selected which contained crop residues that had been decomposing for 20 days but extracts of which were nontoxic to tobacco seedlings. The pH of these were adjusted from 6.7 to 5.0 with HCl. Similarly another series, containing similar crop residues at the same decomposition period except that the extracts in this case were highly toxic, was adjusted from pH 5.0 to 6.7 with NaOH. More plant residue was then added to both series of crocks and allowed to decompose for an additional 10 days. The pH of the contents of these two series of crocks was tested every 3 days adding HCl or NaOH when necessary to maintain their new pH values. At the end of the 10-day decomposition period, aqueous extracts were obtained from both series. It was found that the extracts from crocks which had been highly toxic initially gave rise to products which were only weakly toxic after their pH had been shifted to the less acid range. Extracts from the other series of crocks which had previously given rise to nontoxic products gave rise to toxic products following their adjustment to the more acid range.

Other experiments were carried out using soil which had an initial pH range of 5.0 to 5.4. The various plant materials were added to the soil in the usual manner. To one series of crocks, however, 10 g. of powdered calcium carbonate were added along with the appropriate plant residue. After a decomposition period of 20 days at 60–70° F., the pH and relative toxicity of each of

TABLE V

RELATIVE TOXICITY AS AFFECTED BY DECOMPOSITION OF THE VARIOUS PLANT RESIDUES IN SOILS WITH AND WITHOUT CALCIUM CARBONATE

Plant material added to soil	After 20-day decomposition period	
	pH	Oxygen uptake*
Soil only	5.4	129
Soil+$CaCO_3$	6.7	133
Timothy	4.9	12
Timothy+$CaCO_3$	6.2	146
Rye	5.7	42
Rye+$CaCO_3$	6.8	151
Corn	5.1	28
Corn+$CaCO_3$	6.4	141
Tobacco	5.8	66
Tobacco+$CaCO_3$	6.9	147

*O_2 uptake (in μl. of O_2 after 6 hours at 20° C.) of 50 Harrow Velvet tobacco seedlings. Each figure is an average of four determinations of four replicates each.

the extracts was determined in the usual manner. The results are presented in Table V, from which it is evident that, by maintaining the pH above 6.2 during the decomposition of the residue in question, it is possible to reduce markedly or prevent the formation of substances having inhibiting effects on the respiration of tobacco seedlings. This proved possible even with soils which at first had been quite acid.

The Effect of Aqueous Extracts of Undecomposed and of Partially Decomposed Plant Residues on the Respiration of Tobacco Seedlings

With respect to those toxic substances which may be liberated from the dead or dying plant organs which have been incorporated into the soil it has been shown (2, 3, 21, 23, 27) that many plants contain specific toxic components in certain of their organs which could be released into the soil solution by leaching or disorganization of the tissues. In other instances (11, 21, 28) it has been suggested that the toxicity is due to the intermediate reduction products formed when decomposition of plant residues takes place under certain soil conditions. With many plant residues both of these factors may be operating and it may not always be possible to distinguish between the two sources. Any method which would make it possible to determine relatively rapidly and with some accuracy whether the toxicity was due to unfavorable soil conditions or to specific chemical constituents of the plant in question would help considerably in attempts to modify the detrimental effects. The main purpose of these experiments, therefore, was to determine whether the bio-assay test for toxicity used in this study could also be adapted for this purpose, e.g., to determine whether the plants used in these studies contained specific toxic substances in their tissues or whether the toxic effects were mainly the result of decomposition of the residues under acid or high soil moisture conditions.

In the first series of tests comparisons were made between the effects on respiration of tobacco seedlings produced by exposure of the seedlings to aqueous extracts of soils in which the various plant residues had not or had just begun to decompose and those which had been exposed to extracts from similar soils in which the residues had been decomposing for 15 days. From the results presented in Fig. 2 it is evident that extracts from crocks in which the plant residues had not yet begun to decompose did not contain substances which induced inhibiting effects on the respiration of tobacco seedlings. In many of these tests the disorganization of the plant materials was effected still more completely by thorough maceration in the Waring blendor or by grinding to a fine powder in a Wiley mill before they were added to the soil, but here again no toxic effects were detected in the absence of decomposition.

In other experiments the various soils and plant materials were prepared in the usual manner except that they were placed in large Erlenmeyer flasks and plugged with cotton. One series of flasks was then partially sterilized by autoclaving for 40 minutes at 18 lb. pressure before incubating, whereas the other series was not autoclaved. After both series of flasks had been allowed to incubate for 15 days water extracts were obtained and the relative toxicity of the various extracts was determined in the usual manner. In this

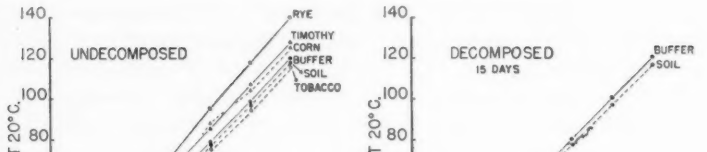

FIG. 2. Comparison of the O_2 uptake of 50 tobacco seedlings as affected by 16 hours' exposure to aqueous extracts of soil (pH 5.3–5.4) to which various plant materials had been added but which had not begun to decompose (undecomposed) and the O_2 uptake of similar seedlings exposed to extracts in which such plant materials had been decomposing for 15 days (decomposed 15 days). In each instance after the exposure period the seedlings were returned to phosphate buffer solution and the O_2 uptake measurements were carried out with the seedlings suspended in the buffer solution. The plant materials added to the soil are indicated in the graphs. Each Warburg vessel contained 2.2 ml. 0.01 *M* phosphate buffer solution (pH 5.3–5.8) and 0.2 ml. 20% KOH in the center well. The O_2 uptake of the buffer solution without the seedlings was negligible and no corrections were necessary.

instance, too, the effects on respiration were similar to those shown in Fig. 2 and autoclaving appeared to delay or inhibit the production of toxic substances. If, however, the autoclaved flasks were kept for periods longer than 15 days many of the soil organisms associated with toxin production apparently became re-established in sufficient numbers to produce some of the toxic decomposition products. Thus, for example, most of the extracts obtained after 25 days reduced the oxygen uptake by as much as 35%. The longer the interval between autoclaving and testing, the greater the degree of toxicity of the resulting extracts, timothy in each instance usually being the most toxic.

Color differences were also observed between extracts obtained from the autoclaved and the nonautoclaved series. After the 15-day period, water extracts of flasks from the autoclaved series were light brown in color while those from flasks which were not autoclaved were usually dark blue to black. The dark color appeared to be characteristic of most of the highly toxic extracts obtained throughout this study. The addition of NaOH (pH 8 to 10) to such extracts resulted in rapid flocculation and precipitation of the pigmented fraction. The toxicity of these extracts, however, was markedly reduced by this treatment. The resuspended precipitate too was only weakly toxic to tobacco seedlings and the toxic principle appeared to be destroyed by such a treatment.

Another difference which was noted between extracts from the autoclaved and the nonautoclaved series was associated with the pH. Whereas most of the extracts from the autoclaved series after the 15-day period were in the pH range of 6.4 to 7.1, those from the nonautoclaved series after the same period were in the 4.9 to 5.7 range. Readjustment of the pH of the extracts from the

autoclaved series to the pH range 4.9–5.7 or, conversely, readjustment of the extracts from the nonautoclaved series to 6.4–7.1 had little effect on their original toxicity.

In the final test of this series the various plant materials were macerated in the Waring blendor or were first air-dried and then ground to a fine powder in a Wiley mill. Water extracts of the plant materials were then obtained without the addition of soil. The pH of these extracts ranged between 6.7 and 7.5 and they were brown in color. After they were centrifuged and filtered the relative toxicity of each of these extracts was determined in the usual manner. Again, the results were similar to those in Fig. 2 and these extracts did not appear to have any inhibiting effects on the respiration of tobacco seedlings.

The results of these tests together with those presented earlier indicate that the tissues of the plants being dealt with in this study, namely, timothy, rye, tobacco, or corn, do not contain substances having inhibiting effects on the respiration of tobacco seedlings. Most of the toxic substances which were obtained appeared to have been formed during the decomposition processes. The total absence of specific toxic components in the tissues of the plants is not completely ruled out, however, since some of these substances might occur in certain tissues of these plants and not be released by leaching with water or maceration of the tissues. It is also possible that such toxic components might occur at stages of the plant maturity other than those tested. From the results presented it may be concluded, however, that the various toxic effects which have been obtained in this study are due mainly to the toxic products arising during the decomposition processes of the various crop residues under the experimental conditions described.

Rate of Penetration, Rapidity of Response, and Persistence of the Inhibiting Effects on Tobacco Seedlings by the Toxic Decomposition Products

The biological effectiveness of the toxic substances under present consideration and the magnitude of their harmful effects on plants would depend not only on their concentration in the soil solution but also on their rate of penetration or absorption by the roots, the persistence of the toxic effects produced by them, as well as on many other factors. Experiments were, therefore, undertaken to determine firstly how rapidly the inhibiting effects on the respiration of tobacco seedlings would become evident following their exposure to the action of the toxic decomposition products, and secondly to determine the persistence of the inhibiting effects upon removal of the seedlings from the toxic extracts.

The general procedures for these tests were similar to those used for the other respiration studies described. In these tests, however, the tobacco seedlings were not pre-exposed to the various extracts for the 16-hour period period prior to the determination of their respiration rates. The oxygen uptake of the seedlings was determined for the first hour in the 0.01 *M* phosphate buffer solution, then the various test extracts were added from the side

arms of the Warburg flasks and the oxygen uptake measurements were continued for an additional 8 hours with the seedlings exposed to the action of the various extracts and the buffer solution. Sterile tobacco seedlings were obtained in the usual manner and the test extracts used were those which, from previous tests, were known to be highly toxic. The Warburg flasks were prepared in the conventional manner with 50 tobacco seedlings per flask. Each Warburg flask contained 1.2 ml. of phosphate buffer solution in which the seedlings were suspended. One milliliter of the various test extracts was then placed in the side arms and alkali was placed in the center well. Thus each of the test extracts was diluted 1.2 to 1 when the two were finally combined in the flasks. The results are presented in Fig. 3 where the oxygen uptake of the tobacco seedlings under the influence of any of the extracts is given as the difference between the oxygen uptake of the flasks containing the seedling and the oxygen uptake of flasks containing the same extracts but without the seedlings. The results were not used, however, if the total oxygen uptake of these flasks without the seedlings exceeded 10 μl. of O_2 per flask during the total test period since this indicated that the extracts in these flasks had not been properly filtered and still contained some microorganisms.

From the results presented it will be noted that the substances present in some extracts were so potent that the inhibiting effects on the respiration of tobacco seedlings could be measured when the seedlings had been in the extracts for less than one hour. The severity of the inhibiting effects on respiration varied in accordance with the plant residue, decomposition products from

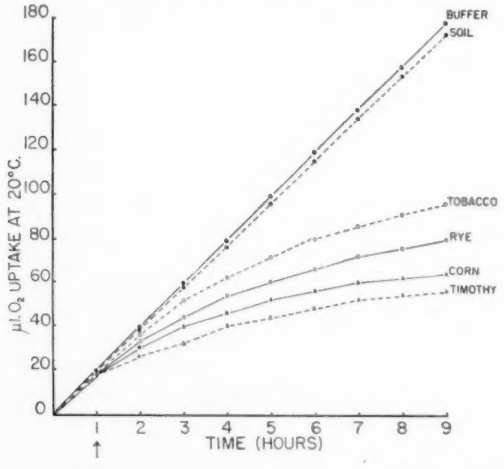

FIG. 3. Oxygen uptake of 50 tobacco seedlings in phosphate buffer solutions to which had been added aqueous extracts of soils in which the various plant materials indicated in the graph had been decomposing for 15 to 25 days. Each Warburg vessel contained 1.2 ml. of the buffer solution (pH 5.3–5.8) in which the seedlings were suspended, 0.2 ml. 20% KOH in the center well, and 1.0 ml. of the various test solutions (pH 5.3–5.8) which were added from the side arms after the 1st hour as indicated by the arrow. Corrections were made for the O_2 uptake of the test solutions without the seedlings.

timothy being the most toxic and those from tobacco showing the least toxicity. This could be due to the differences in the relative concentrations of the toxic components or it could indicate chemical differences between the substances produced during the decomposition of the various residues.

Experiments were next carried out to determine the persistence of the inhibiting effects produced by the 8-hour exposure period to the action of the various extracts. For this purpose the tobacco seedlings from each flask in the experiment above were carefully removed and kept separate. They were rinsed twice in sterile distilled water, returned to the Warburg flasks containing sterile phosphate buffer solution, and their oxygen uptake was again determined. Since this procedure required about two hours the seedlings remained in sterile distilled water for this period of time before their oxygen uptake was again determined. The results are presented in Fig. 4. It will be observed that there was little recovery from the injurious effects produced by the decomposition products of timothy or corn but almost complete recovery from the products of tobacco and rye. These differential rates of recovery and the irreversibility of the injurious effects produced by the decomposition products of some plant residues may be highly significant in connection with the relative severity and type of injury that these substances might induce on plants under field conditions.

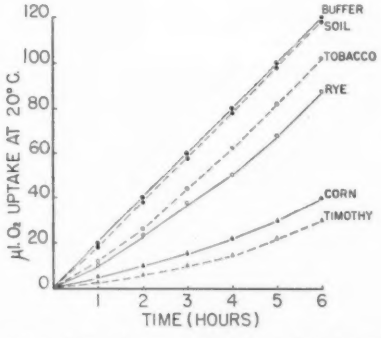

FIG. 4. Oxygen uptake of 50 tobacco seedlings in phosphate buffer. The seedlings were from the experiments described in Fig. 3 and had been exposed for 8 hours to decomposition products of the crops indicated. Each Warburg vessel contained 2.2 ml. 0.01 *M* phosphate buffer solutions (pH 5.3–5.8) and 0.2 ml. 20% KOH in the center well. The O_2 uptake of the buffer solution without the seedlings was negligible and no corrections were necessary.

Effect of the Various Toxic Extracts on the Roots of Tobacco Seedlings

In addition to the marked inhibiting effects on respiration of tobacco seedlings the substances arising during the decomposition of the various plant residues produced other effects. One of the more striking of these was their highly selective effects on the different regions of the root. When tobacco seedlings were placed in some of the extracts the apical meristem region soon turned dark brown (Fig. 6) whereas in other extracts the zone of elongation turned brown while the region of the apical meristem remained white (Fig. 7).

In tobacco seedlings placed in nontoxic extracts, in buffer solution, or in extracts of soil which did not contain decomposing plant residues both of these regions remained relatively white (Fig. 5). A more detailed examination of this phenomenon showed that the effect on the apical meristem was produced mainly by the highly toxic extracts which were obtained when the various plant residues had been decomposing in the soil for 15 to 25 days, while the effects on the region of elongation were obtained with extracts which were somewhat less toxic. In general, those extracts which reduced respiration by 60% or more also produced the browning of the apical meristem region and those which inhibited respiration by 25 to 60% produced the browning of the elongation zone and apparently produced no visible effect on the apical meristem. There were a few exceptions, especially timothy decomposition products which almost exclusively affected the apical meristem and those of rye which affected principally the elongation region. In no instance was browning of either region produced by extracts which did not inhibit respiration.

Microscopic examination of crushed tissue mounts showed that the affected cells of the apical meristem were filled with disorganized masses of brown granular and amorphous material while there was no discoloration in the root cap or the region above the apical meristem. Parallel examinations of roots where the region of elongation was brown showed that the brown pigment was

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FIGS. 5 to 7. These figures illustrate a few of the more striking effects which were observed when tobacco seedlings were exposed to toxic extracts obtained from the decomposition of the various plant residues in the soil. Initially the seedlings used in these tests were identical in appearance, all having been taken from the same composite sample on the 6th day of germination. When such seedlings were exposed for 12 hours to buffer solution or to aqueous extracts of soil which did not contain decomposing plant residues they remained healthy with white roots and numerous root hairs (Fig. 5). When similar seedlings were exposed for the same period of time to aqueous extracts of soil in which residues from timothy, corn, rye, or tobacco had been decomposing for various periods of time, their growth and root hair development was greatly inhibited. The roots were especially sensitive to these substances and visible browning and necrosis of root cells often occurred within 1 hour after exposure. The toxic products were selective in their action and certain of these extracts affected only the cells of the meristematic region (Fig. 6). Browning of this region was induced most frequently by decomposition products from timothy and by extracts which in the other tests had reduced respiration by over 60%. Other extracts, especially those from the decomposition of rye, affected only the cells of the elongation region and the cells of the meristem region remained white (Fig. 7).

FIGS. 8 and 9. Effects similar to those described above were obtained when timothy seedlings were used in place of tobacco and while normal growth and root development were obtained when timothy seedlings were exposed to extracts of unamended soil (Fig. 8) marked inhibition and root injury were obtained when they were exposed to extracts containing toxic decomposition products (Fig. 9). As in the case of tobacco, although all parts of the seedlings were in contact with the toxic extracts the visible injurious effects appeared to be restricted mainly to the region of the root and in most instances root development was partially or completely inhibited (Fig. 9).

FIG. 10. The toxic substances formed during the decomposition of the various plant residues were not specific for tobacco and the same effects were produced when either tobacco, timothy, or barley seed was exposed to their action. Thus when barley seed was placed on filter paper which previously had been soaked in the toxic extracts no germination occurred after 7 days, while those placed on filter paper soaked in water had all germinated by this time. The seed used in this instance had not been surface sterilized and by the 7th day the filter paper and many of the germinating seedlings were covered by fungal growth (top Petri dish); no such fungi were observed on seed which had been exposed to the toxic extracts (bottom Petri dish).

PLATE I

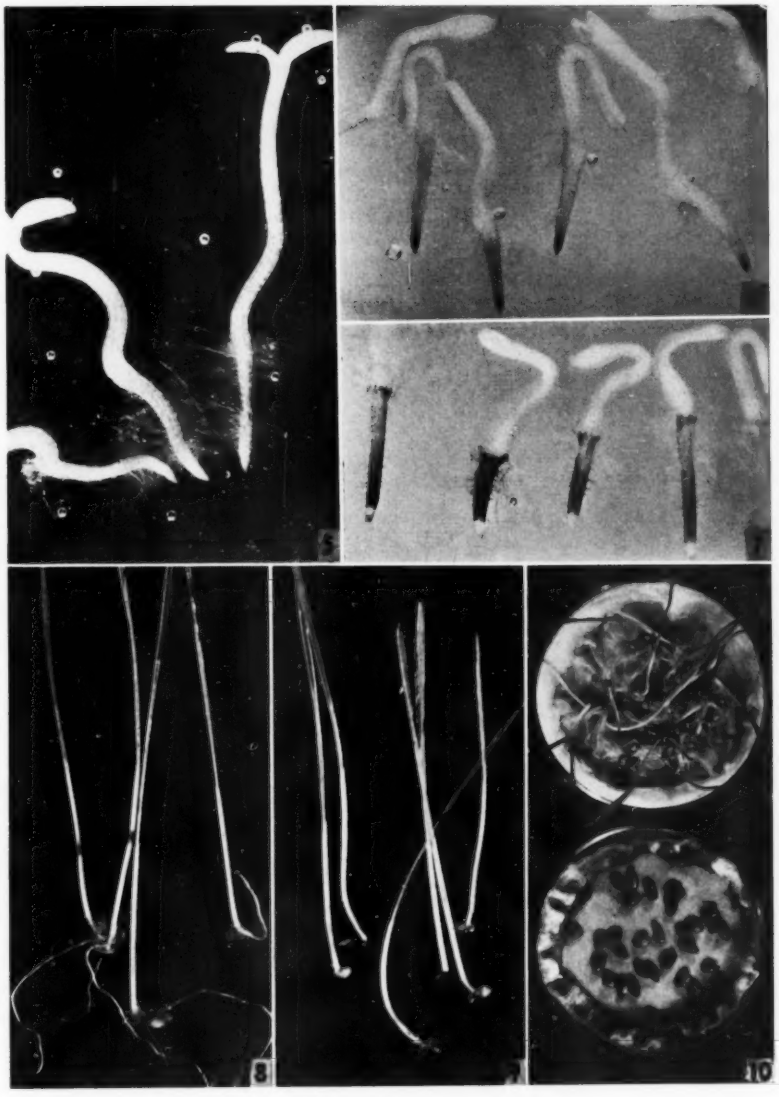

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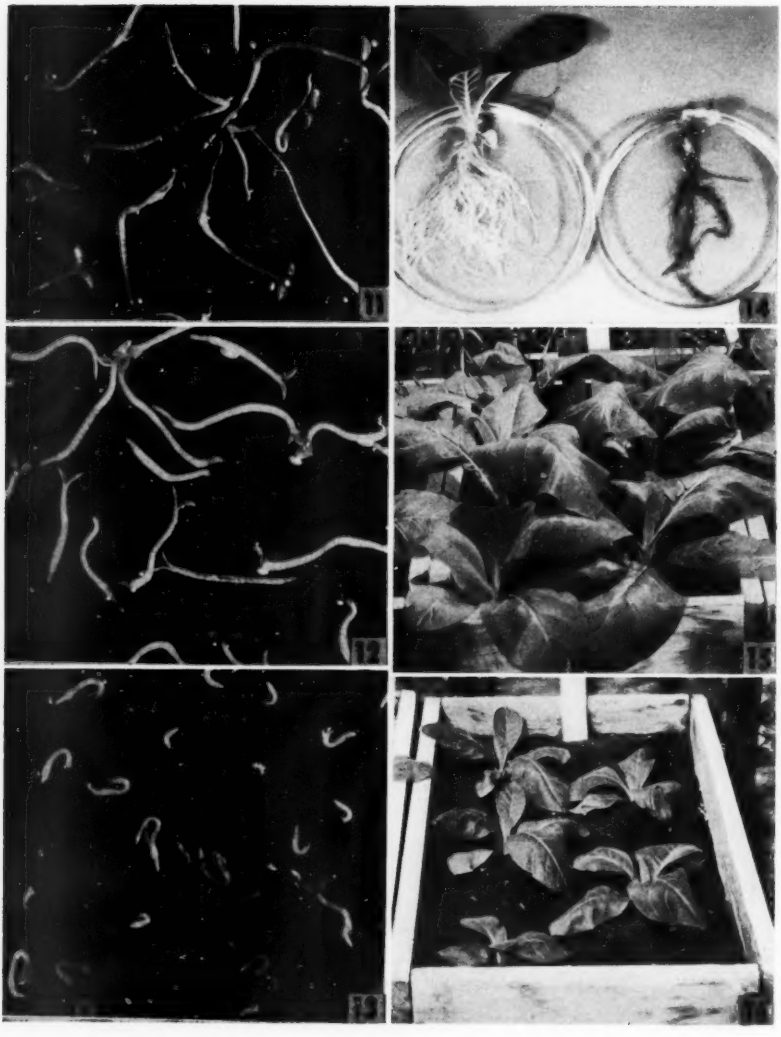
PLATE II



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mainly concentrated in the cell walls, with the epidermal cells being most affected and the discoloration gradually decreasing toward the center of the root.

Other experiments showed that the effect on the apical meristem was quite rapid, that browning could be observed when seedlings were placed for less than one hour in extracts which produced this effect, and that the effect was irreversible when the seedlings were removed from the toxic extracts. Where the browning effect was on the region of elongation discoloration could be noted only in about four to six hours after the seedlings had been exposed to the extracts and again appeared to be irreversible.

All the extracts that possessed other toxic properties also inhibited root hair formation and root hairs were much more abundant on seedlings which had been exposed to extracts of soil only (Fig. 5) than those which had been exposed to the highly toxic extracts containing decomposing plant residues (Figs. 6 and 7). It was also noted that although all parts of the seedlings were in contact with the toxic extracts the visible injurious effects appeared to be restricted mainly to the regions of the root.

In parallel tests carried out with tobacco and timothy seedlings and with excised corn root tips it was found that while timothy seedlings were affected in the same manner as the tobacco by the various extracts, the excised corn root tips, on the other hand, did not show the browning of either the apical meristem or the region of elongation even though their respiration was inhibited.

Effect of Decomposition Products of Timothy, Rye, Tobacco, and Corn on Germination and Growth of Tobacco

Although the respiration of tobacco seedlings was used as the principal assay method for evaluating the relative toxicity of the substances under consideration in these studies, their effects on the germination and growth of

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FIGS. 11 to 13. The effect of the various toxic extracts on germination and growth of tobacco is illustrated. The results were taken after the 7th day and good germination and growth was obtained when the seed had been placed on filter paper previously soaked in water (Fig. 11). Similar and often slightly better germination and growth was obtained when the seed was placed on filter paper soaked in aqueous extracts of unamended soil. When tobacco seed was placed on filter paper soaked in extracts containing decomposition products of rye or tobacco the germination was delayed and growth was inhibited (Fig. 12). Marked inhibition of germination and growth was obtained when the tobacco seed was exposed to decomposition products of timothy or corn (Fig. 13); in many instances germination of tobacco, timothy, or barley seed was completely inhibited by such extracts.

FIG. 14. Severe wilting and rotting of roots of older tobacco plants were also readily induced by many of the toxic decomposition products. These injurious effects were readily demonstrated in plants growing in nutrient culture in quartz sand. The plant on the right had received one application of the toxic decomposition products from timothy. Severe wilting was obtained 24 hours after the application and discoloration and rotting of the roots was obtained 3 days after the application of the toxic extracts.

FIGS. 15 and 16. Stunting of tobacco plants was obtained in the greenhouse when materials from timothy, rye, tobacco, or corn plants were added to flats of soil and allowed to decompose for 21 days before tobacco was planted. Good growth was obtained in flats to which no plant residue had been added (Fig. 15). Plants growing in flats to which timothy or corn residue had been added were severely stunted (Fig. 16). The stunting was less severe in flats to which rye or tobacco residue had been added.

TABLE VI

EFFECT ON GERMINATION AND GROWTH OF TOBACCO AFTER 7 DAYS' EXPOSURE TO THE VARIOUS EXTRACTS

Type of test extract	Decomposition period (days) at 60–70° F.						
	0	5	10	15	20	25	30
Water (check)	225*	210	195	210	205	210	203
Soil	240	215	202	242	237	245	208
Soil+timothy	215	82	60	55	65	100	85
Soil+rye	227	110	72	82	127	107	130
Soil+corn	213	123	135	65	85	68	98
Soil+tobacco	232	164	142	105	132	205	200

*Total length in mm. of 25 tobacco seedlings after 7 days' incubation at room temperature (70–80° F.). Each figure represents the averages of eight different decomposition series in which tests with each extract were replicated four times.

tobacco, barley, and timothy were also determined as a check on the reliability of the basic technique and to determine the range of activity of these substances. The method was described in some detail earlier and most of the extracts on which respiration studies were carried out were also tested by this method.

The results are summarized in Table VI from which it will be noted that the relative toxicity of the various extracts as determined by this method was reasonably similar to that in which respiration was used as the test response. In this method the relative toxicity is shown as the length in millimeters of 25 germinating tobacco seedlings after 7 days. Thus, the extracts which were highly toxic as determined by the effects on respiration were also the most toxic as determined by the latter method. It appears, therefore, that the substances which are produced during decomposition of the various plant residues can affect respiration, germination, and growth. In these tests no germination of tobacco seed occurred even after 15 days in extracts which had inhibited respiration by 70% or more. In other extracts which had less pronounced effects on respiration the effects on germination and growth were also less pronounced and ranged from germination after only 3 days followed by rapid growth to rapid germination followed by slow growth with necrosis of the radicles and to delayed germination with little or no further growth. As with respiration, these effects too were influenced by the type of plant residue added to the soil, the decomposition period, as well as by the other factors dealt with previously. Again decomposition products of timothy were most toxic while those of tobacco showed the least toxicity.

Additional Physical and Chemical Properties of the Toxic Decomposition Products

A few additional tests were carried out with the highly toxic extracts to determine their specificity, solubility, stability to heat and in storage, and other properties.

To test the specificity of the toxic extracts a series of tests were carried out to determine whether the toxic effects on germination and growth of tobacco

seed would also be produced when barley or timothy seed were used. These tests were carried out simultaneously and the effects on the three types of seed by the various toxic extracts were compared. Comparisons were also made of the effects on the respiration of tobacco seedlings and on excised corn root tips and almost identical results were obtained with each. From the results obtained it appears that the toxic substances present in the various extracts were relatively nonspecific in their action, affecting tobacco, timothy, and barley in approximately the same manner (see Figs. 5–10).

During many of the tests it was noted that all of the toxic extracts also had some antifungal properties. This was most clearly illustrated in tests in which the seed used had not been surface-sterilized. In such cases it was noted that when the seeds were placed on filter paper soaked in the nontoxic extracts or in water they were soon covered with the fungi which normally are found on the surface of these seeds. Germination and growth of tobacco and barley seed did not appear to be appreciably affected by most of these organisms during the 7-day test period. Seed placed on filter paper soaked in the toxic extracts remained completely free of fungi even though they contained seed from the same sample (Fig. 10). Tests to determine the species of fungi which could be inhibited by these extracts have not been completed.

In preliminary isolation, purification, and concentration tests it was found that the toxic substances were soluble in water and insoluble in petroleum ether. They were stable in acid and were most active in the acid range pH 4.5 to 5.8. They were inactivated by alkali and did not regain their original activity when readjusted to the acid range. These substances were also found to be heat stable. Boiling for ½ hour or autoclaving for 20 minutes at 16 lb. pressure at pH of 4.0 to 5.5 did not appreciably reduce their toxicity. The toxicity of the extracts was not affected on prolonged storage at 1° to 3° C. provided they were covered by a layer of toluene. The toxicity was somewhat reduced after the extracts had passed through activated charcoal.

Greenhouse Experiments

A few preliminary experiments were carried out in the greenhouse with older tobacco plants to determine whether certain of the toxic effects could be observed under greenhouse conditions. In one series of tests, which will be described briefly, the various plant residues were added to a Fox sandy loam soil obtained from the Delhi tobacco growing area. This soil was placed in deep flats, well fertilized, and the plant material was allowed to decompose for 21 days under normal moisture conditions. There were four replicates for each of the plant residues used and flats of the same soil to which no plant residues had been added served as the controls. After a 21-day decomposition period five tobacco plants each 6 in. in height were planted in each of the flats and results were determined 15 days after planting. Severe stunting was obtained in flats to which timothy or corn had been added. Less stunting was obtained in flats where rye or tobacco had been added and good growth was obtained in flats which did not contain crop residues (Figs. 15 and 16).

In other experiments tobacco was grown in quartz sand in which some of the plants received nutrient solution while others received both nutrient solution and the highly toxic extracts. In many instances severe wilting was obtained 24 hours after the application of the toxic extracts. The roots of the plants receiving the highly toxic extracts had also turned black and rotted within 3 days after the application of the extracts (Fig. 14). Applications of the toxic extracts were also made to well-established tobacco plants growing in soil. In this instance, however, some wilting was observed only after the fifth application, suggesting that much of the toxic component was adsorbed by the soil colloids and much larger quantities of the extracts had to be added before some plant response became evident. More comprehensive greenhouse and field experiments will be necessary, however, before the precise significance of the various toxic effects observed here under laboratory conditions can be fully evaluated and related to conditions in the field.

Discussion

The present studies have shown that substances possessing phytotoxic properties may be formed during the decomposition of plant residues in the soil. This confirmation of the production of phytotoxic compounds during the decomposition of residues is of special interest because of the suspected importance of such compounds in the etiology of certain root rots (10, 13, 17, 18, 24). It is with these toxic compounds, therefore, that the present studies have been primarily concerned. An attempt has been made to elucidate some of the factors concerned in their formation, to evolve suitable methods for their assay, and to study some of the harmful effects that such substances have on plants in the absence of other causal factors.

Since it was quite evident that most studies dealing with the question of whether or not decomposition of plant residues in the soil can give rise to substances toxic to plants have been seriously handicapped by the lack of reliable and rapid assay methods, concerted efforts were made to approach the problem on a more precise qualitative and quantitative basis. With this purpose in mind the possibility of adapting the Warburg respirometer for the purpose of studying certain phases of the problem was thoroughly explored. By using inhibition of respiration as an index of toxicity it was possible to determine some of the conditions under which the decomposition of plant residues in the soil gave rise to phytotoxic by-products and to obtain an accurate measure of the relative toxicity of such substances. Thus, certain relationships between the nature of plant residue added to the soil, the decomposition conditions, and the degree of toxicity of the resulting products could be readily determined. If on wider application and further study this bio-assay method should continue to be as satisfactory as in the current investigation it would offer considerable promise in overcoming many of the difficulties encountered in such studies.

Since these have been laboratory experiments, the interpretation as to the possible significance of the results presented must be viewed in the light of

this obvious limitation. Whether the toxic decomposition products obtained in this study can also be produced under field conditions or affect the plants in the manner indicated in such a complex system as the soil environment cannot be definitely stated at this time. In view of the constant occurrence of plant residues as one of the normal soil constituents, however, and since the toxic substances dealt with in this study were obtained quite readily under conditions of decomposition which might also pertain at certain times in the field, the possibility that similar substances may be produced and released into the soil solution is not too remote. It may be true that, although conditions most favorable for the production of toxic products are most likely to occur in poorly drained and acid soils, the production of such substances is not limited to such soils. Since in these experiments they could be produced relatively readily in all of the soils tested, many of which initially were only weakly acid and only temporarily saturated, it is possible that their occurrence may be more common than is generally realized. It is possible, therefore, that toxic decomposition products may occur in soils and under conditions which when considered as a whole would not appear to favor their production. In such instances these substances are likely to be produced in localized areas where the concentration of plant residues, water content, pH, and other factors are favorable for the type of breakdown leading to toxin formation. There is no doubt that most of these substances, no matter how toxic they are, eventually disappear from the soil (2). Regardless of the period of existence of such compounds, however, because of their marked toxicity and rapidity of action they could, nevertheless, induce important physiological and morphological changes in the plant. Owing to the nonspecific nature of their action and the difficulty of their detection, many of the harmful effects would often be attributed to some other more obvious causal factors. By using the more precise and sensitive assay methods, however, it has been clearly demonstrated that many of these substances can exert injurious effects in the absence of other causal factors. It is significant, too, that the roots appear to be especially sensitive to these products. It may be possible that the index used to demonstrate and evaluate toxicity is not a true reflection of the over-all phytotoxic nature of the products involved, since the significance of many of the respiratory changes in plants is not clearly understood (1, 7). There is a strong likelihood, however, that any condition which gives rise to a reduction in the respiratory activity is also likely to result in some retardation of all the physiological functions, with the corresponding inhibiting effects on many of the processes associated with growth and development. Combined with the inhibitory effects on respiration the same extracts induced marked injury to root cells and either completely inhibited or retarded germination and growth. These results suggest, therefore, that in addition to the more readily visible injurious effects many of the toxic decomposition products are also likely to exert less obvious but perhaps more significant changes in the living plant. Many of these are likely to be expressed indirectly as a deficiency, a stunting, or more commonly a root-rot condition produced by organisms whose pathogenicity often cannot be conclusively demonstrated.

Therefore, in view of their ability to produce such a broad range of injurious effects in the absence of other causal factors, it is highly probable that toxic decomposition products may be performing a significant role in the soil not only as primary causal and initiating agents of certain root rots but also in predisposing the plant, especially the root system, to invasion by organisms not normally regarded as pathogenic. In the light of the data presented and other information available (12, 13, 24, 29) it is evident that in a consideration of almost any root-rot disease of plants the important role of such products in the disease complex cannot be overlooked.

Acknowledgments

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SEASONAL CHANGES IN METABOLISM OF SPRUCE LEAVES[1]

A. C. NEISH

Abstract

Sucrose, raffinose, pinitol, shikimic acid, and pungenin were isolated in crystalline form from leafy twigs of both Colorado spruce (*Picea pungens* Engelm.) and white spruce (*Picea glauca* (Moench) Voss) collected in winter. Raffinose and sucrose were the main sugars present in winter but raffinose disappeared in early spring and was not found again until autumn. Pungenin was also found in lowered concentrations in summer, and pinitol could not be crystallized from material collected in warm weather.

Leafy twigs were harvested at different seasons and allowed to photosynthesize in the presence of $C^{14}O_2$ under constant conditions in the laboratory. Winter twigs were found to be convenient and efficient for the preparative biosynthesis of sucrose-C^{14} and raffinose-C^{14}. Hemicellulose was labelled much more rapidly than cellulose. The C^{14} in the hemicellulose was concentrated in the glucose, little being found in the xylose.

The relative rates of labelling of the sugars, pinitol, shikimic acid, pungenin, cellulose, and lignin showed some marked seasonal changes.

Introduction

This work was originally started as an investigation of the usefulness of spruce cuttings for the preparative biosynthesis of C^{14}-labelled compounds from $C^{14}O_2$. During the severe Canadian winters conifer leaves are the only material available from natural sources for photosynthetic experiments of this type. It is known that these leaves are able to carry on photosynthesis in cold weather (14, 31) although the rate becomes quite slow at temperatures below $-6°$ C. (14). In the present work experiments have been done with leafy twigs, collected at various seasons of the year, and studied under constant conditions in the laboratory.

Severing of twigs from the branches does not immediately affect the rate of photosynthesis (10). The tips of the branches should give the best cuttings since leaves formed during the previous summer are reported to be the most active in photosynthesis (15).

Spruce leaves are known to contain a number of simple compounds which may be isolated in pure form. These include sucrose (32), shikimic acid (20), and pinitol (32). In addition a glucoside of 3,4-dihydroxyacetophenone (pungenin) has been identified as a major constituent of the leaves of Colorado spruce (30). Raffinose has also been found in conifer seeds (19) although some question has been raised about its occurrence in spruce leaves (2). The isolation and rigorous identification of all of these compounds in both Colorado spruce (*Picea pungens* Engelm.) and white spruce (*Picea glauca* (Moench) Voss) is described in this communication.

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Colorado spruce was readily accessible to the author and was used exclusively in the photosynthetic experiments described below. The leafy cuttings were treated with $C^{14}O_2$ under conditions suitable for photosynthesis and then given an additional period of photosynthesis in ordinary air. The compounds mentioned above were then isolated, purified, and their specific activities measured. Experiments were performed with twigs collected at all seasons of the year. It was found that spruce cuttings collected in winter can be used conveniently and efficiently for the preparation of sucrose-C^{14} and raffinose-C^{14} but the other compounds were labelled too slowly to be prepared efficiently in this way.

There is another aspect to this work which may be of more interest to the biologist than the preparation of C^{14}-labelled compounds. The specific activities of the compounds isolated are an index of their rate of formation. From the data collected it was possible to compare rates of biosynthesis in twigs collected at different seasons of the year since they were all tested under constant laboratory conditions. In other words seasonal changes in internal factors governing metabolism of spruce twigs were detected. The advantage of the tracer technique for this purpose lies in the fact that biosynthetic rates can be measured in substances not showing any marked changes in concentration, during the experiment.

Experimental

Treatment with $C^{14}O_2$

A simple apparatus, which was found to be convenient for effecting the photosynthetic assimilation of $C^{14}O_2$ by spruce twigs, is shown in Fig. 1. The lights were set in opposition at the proper distance (about 10 in.) from F to give an intensity of 1000 ft-c. inside G. If the temperature of the water in F was kept at 15–16° C. the air temperature in G was 22–23° C. This was accomplished by a coil of copper tubing (not shown), with cold water flowing through it, immersed in F. In wintertime Saskatoon tap water was cold

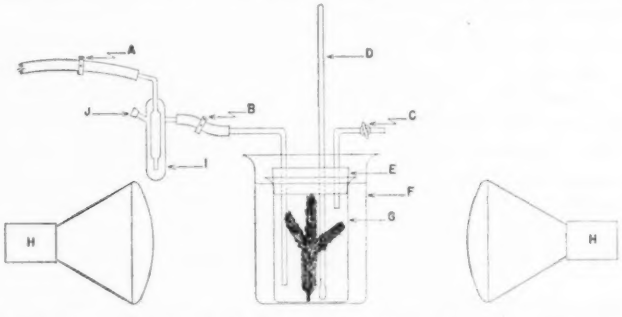

FIG. 1. Apparatus used for $C^{14}O_2$ treatment. A and B—hosecocks, C—stopcock, D—thermometer, E—rubber stopper, F—2000 ml. Pyrex beaker containing water, G—600 ml. Pyrex beaker (tall form) containing spruce twigs, H—300-w. photoflood incandescent light, I—Pyrex bubbler (20 ml. capacity), J—serum bottle cap.

enough for this purpose but in summer the water in the coil had to be circulated from a refrigerated bath. The temperature in G was set by adjusting the rate of flow of coolant through the copper coil. The coil was arranged so it did not shade the twigs.

In a typical experiment G was rinsed with water and two twigs (total fresh weight 20–30 g.) were put in it while the walls were still damp. The bubbler I was charged with 1 ml. of $Na_2C^{14}O_3$ solution (0.2 mM.). With B and C open and A closed the apparatus was evacuated through C to about 40 mm. Hg. pressure; then B and C were closed and 1 ml. of 10 *N* sulphuric acid was injected through J using a hypodermic syringe fitted with a stainless-steel needle. The hosecock B was opened cautiously and the whole appartus was then brought to atmospheric pressure by letting air in through A, thus sweeping all of the $C^{14}O_2$ into G; B was then closed and I removed. The twigs were illuminated at 1000 ft-c. and 22–23° C. for 5 hours. The small amount of residual $C^{14}O_2$ was then swept out through C, by air entering at B, into a bead tower containing dilute NaOH. The contents of the tower were then analyzed for C^{14}. It was found that in 3 hours about 65% of the carbon dioxide was assimilated by the twigs, while in 5 hours over 99% was taken up.

The final sweeping occupied about one hour. The twigs were fractionated immediately or put in an illuminated incubator where they were allowed to metabolize further in ordinary air at 22° C. while receiving 400 ft-c. from "cool white" fluorescent tubes.

Fractionation of Spruce Twigs

The initial stages of the fractionation are outlined in Fig. 2, which is largely self-explanatory. Evaporations were accomplished by an air stream at room temperature. "Hot" solvents were heated to the boiling point just before use but were not heated further when in contact with the plant material. In making the hot-water extraction enough Celite was used to ensure a rapid filtration. This relatively large amount of Celite was mixed in intimately, with gradual addition of water at first, to spread the sticky plant extract over the surface of the Celite and thus permit efficient extraction. The volume of the hot-water extract (fraction A) was usually 6–8 ml. per gram based on the original fresh plant material.

Fraction A is composed of salts, sugars, organic acids, pungenin, and other water-soluble constituents. Fraction B contains plastid pigments, fats, waxes, and compounds with a similar solubility. Fraction C is made up of denatured protein, cellulose, hemicellulose, lignin, and possibly other polymeric substances.

These fractions were all analyzed for C^{14}. Fraction B was not investigated further but fractions A and C were examined as described below.

Examination of Fraction A

This fraction (2 g.) was dissolved in water (50 ml.) and the solution deionized by a column containing 20 ml. of Amberlite IR-120 resin (acid form) packed

on top of Amberlite IR-4B resin (basic form), with a disk of filter paper separating the two resins. The column was saved for recovery of the acids.

The deionized solution was evaporated to about 10 ml. volume and put on a 3.8 × 40 cm. charcoal column, prepared from equal volumes of Darco-G-60 and Celite 545 slurried in water. The sample was washed in with water and the column developed by linear gradient elution (3) using water (1500 ml.) balanced with 60% ethanol. Fractions of about 50 ml. were collected automatically and tested for sugars by spotting on paper and spraying with a *p*-anisidine hydrochloride reagent modified for detection of sucrose and raffinose as well as monosaccharides. This reagent was prepared by dissolving 2.5 g. of *p*-anisidine in a mixture of 10 ml. of concentrated hydrochloric acid and 100 ml. of glacial acetic acid. The sprayed papers were heated at 105° C. The colors obtained were those expected for glucose or fructose or mixtures of these sugars (22). The charcoal column gave a good separation of the sugars into a monosaccharide, disaccharide, and trisaccharide fraction as expected (36). The column was developed further with ethanol–benzene, as described previously (30), to give a pungenin fraction.

The monosaccharide fraction gave a sirup on evaporation. Paper chromatograms of this fraction, developed by *n*-butanol:acetic acid:water (4:1:1.8) or *n*-butanol:pyridine:water (6:4:3), showed spots after they were sprayed with the *p*-anisidine reagent which corresponded in position and color with

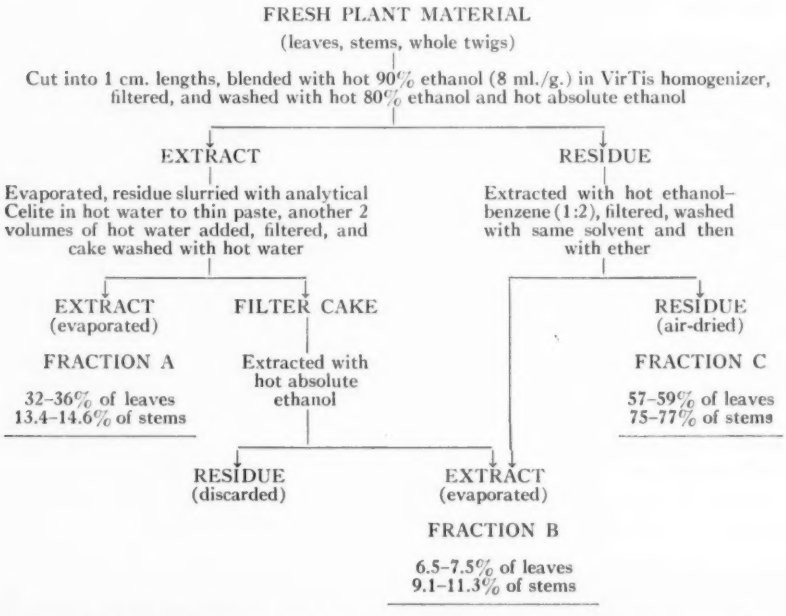
FRESH PLANT MATERIAL
(leaves, stems, whole twigs)
Cut into 1 cm. lengths, blended with hot 90% ethanol (8 ml./g.) in VirTis homogenizer, filtered, and washed with hot 80% ethanol and hot absolute ethanol
EXTRACT
RESIDUE
Evaporated, residue slurried with analytical Celite in hot water to thin paste, another 2 volumes of hot water added, filtered, and cake washed with hot water
Extracted with hot ethanol–benzene (1:2), filtered, washed with same solvent and then with ether
EXTRACT (evaporated)
FILTER CAKE
RESIDUE (air-dried)
FRACTION A
32–36% of leaves
13.4–14.6% of stems
Extracted with hot absolute ethanol
FRACTION C
57–59% of leaves
75–77% of stems
RESIDUE (discarded)
EXTRACT (evaporated)
FRACTION B
6.5–7.5% of leaves
9.1–11.3% of stems

FIG. 2. Initial stages in fractionation of spruce twigs. Yields are based on dry weight.

glucose and fructose. Treatment of the bulk of the fraction with phenylhydrazine gave osazone which was purified by recrystallization from 50% ethanol. This osazone was identical in crystal form with phenylglucosazone. It was used for determination of C^{14} in glucose + fructose. Analysis by the anthrone reagent (28) showed that glucose and fructose make up only 40–50% of the non-volatile matter in the monosaccharide fraction. The filtrate from the osazone was deionized as above and then concentrated to 5 ml. and chromatographed on a 1.1 × 11 cm. charcoal–Celite column using water as the developing agent. The effluent contained a substance which reacted with a periodate–permanganate spray reagent (26). Concentration of the fractions containing this substance gave a white crystalline material after treatment with ethanol. This was recrystallized by dissolving in a small amount of water and adding ethanol; m.p. 188° C.; $[\alpha]_D^{25} = 64.6°$ ($c = 1$, water); 15.90% methoxyl. These constants agree with those of *d*-pinitol. A mixed melting point with an authentic sample of *d*-pinitol (obtained from Dr. J. A. F. Gardner) showed no depression and the X-ray diffraction powder diagrams and infrared spectra of the two samples were identical.

The disaccharide and trisaccharide fractions were examined by paper chromatography as described above. Each contained a single sugar with the mobilities of sucrose and raffinose respectively. Analysis by the anthrone method showed that practically all the non-volatile matter in these fractions was carbohydrate. The sirups obtained on evaporation crystallized readily when treated with ethanol. The crystalline sugars were recrystallized by adding ethanol to their concentrated aqueous solutions. The sugar from the disaccharide fraction, m.p. 190° C., was proved to be sucrose by a mixed melting point determination with an authentic sample. The trisaccharide isolated, $[\alpha]_D^{22} = 104.2°$ ($c = 1.36$, water), was proved to be raffinose by comparison of the X-ray diffraction powder diagram with that of an authentic sample.

Hydrolysis of sucrose (300 mg.) was effected by 2 ml. of 0.75 *N* hydrochloric acid, 10 minutes at 60° C. The acid was removed by IR-4B resin, the neutral filtrate evaporated, and the glucose and fructose separated on a 2.9 × 33 cm. cellulose column using phenol–water (4).

Pungenin was purified by recrystallization first from ethanol–hexane and then from water (30). The identity of the sample from white spruce $[\alpha]_D^{23} = -97.8°$ ($c = 0.5$, water), was proved by a mixed melting point with an authentic sample from Colorado spruce, and by hydrolysis to 3,4-dihydroxyacetophenone, which was also identified by a mixed melting point determination.

The water-soluble acids were recovered from the IR-4B resin used in the initial deionization. The IR-120 layer was removed from the composite column and discarded. A solution of *N* sulphuric acid was passed through the IR-4B layer remaining in the tube, until the effluent gave a strong test for sulphate. Another 25 ml. was collected, the combined eluates neutralized by barium carbonate, filtered with analytical Celite, and the filtrate put through a small column of IR-120 to remove barium ions.

The acidic filtrate was evaporated and put on a 2.1 × 20 cm. charcoal–Celite column. The sample was washed in with water and the column developed by linear gradient elution (3) with water balanced by 150 ml. of 50% ethanol. The fractions (3 ml.) were collected automatically and tested for shikimic acid by spotting on paper and spraying with the periodate–permanganate reagent (26). This reagent reacts instantaneously with shikimic acid.

Shikimic acid was usually found in tubes Nos. 45 to 55. The combined fractions were evaporated and the residue recrystallized from glacial acetic acid to give colorless crystals, m.p. 189–190° C., showing no depression in melting point when mixed with an authentic sample of shikimic acid. This acid was also identified as the crystalline benzyl ammonium salt, $[\alpha]_D^{22} = -115°$ ($c = 0.6$, water). This rotation agreed fairly well with that of an authentic sample ($-112°$, $c = 1.0$, water) but neither sample exhibited the sharp melting point previously reported (25).

The acids eluted from the charcoal column before shikimic acid were investigated by paper chromatography using *n*-butanol:acetic acid:water (4:1:1.8) and the benzyl alcohol solvent described by Hathway (18). The sheets were dried and sprayed with either (*a*) 0.1% of sodium 2,6-dichlorobenzenone indophenol in ethanol (1), (*b*) the periodate–permanganate reagent (26), or (*c*) alkaline silver nitrate (34). The acids not adsorbed strongly by charcoal contained a major component which was identical with quinic acid in these tests. Another acid, moving at about half the rate of quinic acid in the benzyl alcohol solvent, was also present. There was no sign of succinic, citric, malic, or oxalic acids. However, phosphate esters were detected on sheets sprayed with a molybdate reagent (17) and exposed to ultraviolet light. An attempt to crystallize quinic acid from the mixture was unsuccessful. Quinic acid is known to be a major constituent of spruce seedlings (24).

Examination of Fraction C

This fraction was tested for starch qualitatively. Ten milligrams were suspended in 1 ml. of hot water; the mixture was then cooled and 0.2 ml. of 0.02 *N* iodine in 0.8% aqueous potassium iodine added. Holocellulose was prepared by three successive chlorite treatments and fractionated by extraction with 10% KOH into cellulose and hemicellulose (23).

A hemicellulose fraction was recovered by acidification of the alkaline extract with acetic acid followed by precipitation with three volumes of ethanol. This was the hemicellulose fraction which was analyzed for C^{14}. It was hydrolyzed by 3% nitric acid, 4 hours at 100° C. (21). The acid was removed by IR-4B resin and the monosaccharides in the hydrolyzate were isolated by chromatography on a charcoal–Celite column. The monosaccharide fraction was examined by paper chromatography. The main sugars appeared to be glucose and xylose. Chromatography on a cellulose column, using *n*-butanol:*n*-propanol:water (4:1:1) as the developing solvent, gave two fractions, one containing only glucose and the other xylose. These fractions were again purified by charcoal column chromatography before analysis for C^{14}.

Crude protein was calculated from Kjeldahl N × 6.25. In order to obtain the amino acid carboxyl as carbon dioxide samples of fraction C (200 mg.) were refluxed with 6 *N* hydrochloric acid (80 ml.) for 24 hours. The hydrolyzate was filtered and the filtrate concentrated first in a Craig evaporator (11) and then by an air stream, to remove most of the hydrochloric acid. The pH was then adjusted to 3 and the solution treated with ninhydrin (16). The carbon dioxide released was condensed by liquid nitrogen and its specific activity measured (6).

Vanillin, from lignin, was obtained by alkaline nitrobenzene oxidation of a portion of fraction C. The vanillin was isolated and its specific activity measured as described previously (5).

C^{14}-Measurements

The C^{14} in fraction C was measured by an end-window (micromil) proportional counter using "infinitely thick" samples. The C^{14} in all other samples was determined by the method of Buchanan and Nakao (6), after conversion to carbon dioxide.

Results

Composition of Spruce Twigs

Table I gives the amounts of some of the water-soluble constituents found in spruce twigs collected in winter. The sugars were determined, by analysis of the appropriate fraction from the charcoal column, using the anthrone method (28). The other compounds were estimated by weighing the first crop of crystalline material obtained in each case.

Raffinose and sucrose were the main sugars present. There is a suggestion in these data that the ratio raffinose/sucrose may vary throughout the winter.

TABLE I

WATER-SOLUBLE CONSTITUENTS OF WINTER SPRUCE TWIGS

	Percentage of dry weight			
	Colorado spruce			White spruce
	Harvested Dec. 11, 1956	Harvested Jan. 14, 1957		Harvested Jan. 14, 1957
Constituent	Whole twigs*	1955 leaves	1956 leaves	Whole twigs*
Glucose + fructose	1.8	1.5	1.1	2.0
Sucrose	2.8	2.1	1.6	2.5
Raffinose	2.5	lost	3.2	4.1
Pungenin	4.3	4.3	3.0	3.1
Shikimic acid	1.6	1.3	1.3	1.0
Pinitol	0.6	ND†	ND	0.4

*The twigs contained 43–45% of dry matter and 75–80% of this was in the leaves; about 80% of the leaves were formed in 1956.

†ND = Not determined.

No marked differences in composition were found between Colorado spruce and white spruce.

As expected from previous work (12), starch could not be detected in leafy twigs harvested in winter, even after 6 hours' photosynthesis at room temperature. A small amount was present in cuttings which had been kept 3 days at 22° C. in light (400 ft-c.). Fraction C of the leaves contained 10.6–11.4% protein, 47–50% cellulose, and 7.8–10.3% of hemicellulose. These analyses are the ranges found for leaves from three lots of twigs kept in light at room temperature for 6, 26, and 72 hours.

Table II shows some seasonal variations in the composition of Colorado spruce leaves. One of the most striking changes was the disappearance of raffinose with the onset of warm weather. It was not found again until autumn. This may explain why Bidwell, Krotkov, and Reed (2) were unable to find raffinose in spruce leaves, since their material was collected in June.

TABLE II

SEASONAL VARIATION IN THE COMPOSITION OF COLORADO SPRUCE LEAVES

Date (1956)	Material examined	% of dry weight*					
		Glucose + fructose	Sucrose	Raffinose	Pungenin	Shikimic acid	Pinitol
May 2	1955 leaves	ND	3.7	1.5	3.8	0.7	1.7
May 22	1955 leaves	ND	3.8	0.3	3.3	1.0	1.3
June 3	1955 leaves	ND	4.2	nil	3.7	0.9	0.4
June 11	1955 leaves	ND	3.3	nil	1.8	1.0	—
	New growth	ND	2.0	nil	—	1.3	—
July 24	1955 leaves	ND	4.3	nil	1.8	1.9	—
	New growth	ND	3.2	nil	1.1	1.3	—
Aug. 27	1955 leaves	ND	4.4	nil	1.0	1.1	—
	New growth	ND	3.2	nil	2.9	1.1	—
Oct. 4	1955 leaves	4.4	5.7	1.2	1.9	—	—
	New growth	1.2	5.1	1.1	2.9	1.3	1.1
Nov. 8	1955 leaves	1.9	3.1	1.0	1.7	—	—
	New growth	1.6	3.8	2.2	5.4	0.6	1.0
Dec. 11	New growth	1.9	3.0	2.6	4.5	1.7	0.6

*ND = Not determined quantitatively; a dash indicates the compound could not be crystallized by the method employed.

The concentration of pungenin was also considerably reduced in warm weather. This agrees with observations made by Tanret (33) on a related glucoside, picein, found in Norway spruce (*Picea excelsa* Link) leaves. He found that picein was present in larger amounts in February than in May (in France). Shikimic acid and sucrose did not show any marked changes in concentration. Pinitol could not be crystallized from material harvested in warm weather. In spite of this there were large amounts of material in the "monosaccharide" fraction (from the charcoal column) with properties quite similar to those of pinitol, on paper chromatograms.

Experiments with $C^{14}O_2$

Twigs collected in the middle of winter were used first (Table III). Most of the C^{14} assimilated by photosynthesis was found in the leaves (Table III, footnote). During subsequent photosynthesis in ordinary air some of this was translocated to the stems but even after 72 hours the major portion of the C^{14} remained in the leaves. Most of the C^{14} in the leaves was present as water-soluble substances (fraction A). These were gradually converted to the insoluble substances making up fraction C, and this conversion was maintained during the 3-day period.

TABLE III

DISTRIBUTION OF C^{14} IN COLORADO SPRUCE TWIGS* (FEB. 1956)

	Percentage of total C^{14} found in:					
	Leaves			Stems†		
	6 hr.	26 hr.	72 hr.	6 hr.	26 hr.	72 hr.
Fraction A	82.8	75.9	61.0	71.5	90.5	77.2
Fraction B	3.8	6.9	4.8	4.7	2.0	4.6
Fraction C	13.4	17.2	34.2	23.8	7.5	18.2

*Photosynthesis in $C^{14}O_2$ for 5 hr. followed by additional photosynthesis in ordinary air to make up the metabolic period indicated.

†The stem contained 0.8, 2.8, and 8.7% of the total C^{14} in the twigs after metabolic periods of 6, 26, and 72 hr. respectively.

Further investigations of these fractions (Table IV) showed sucrose had the highest specific activity of any soluble constituent of the leaves isolated following photosynthesis in $C^{14}O_2$. During subsequent metabolism in ordinary air at 22° C. and 400 ft-c. the specific activity of the sucrose decreased while the C^{14} in other constituents, notably raffinose, glucose, fructose, and hemicellulose, increased. This agrees with the concept that sucrose is the first stored product of photosynthesis (7, 8, 9, 35) and the other carbohydrates are formed from it.

The sucrose was labelled equally in the glucose and fructose moieties as expected for a "long-term" experiment (8). The glucose isolated from the hemicellulose fraction was much more active than the xylose from the same fraction. Cellulose was formed at a very slow rate compared with hemicellulose. There was a parallelism in the rate of formation of pungenin–aglycone and shikimic acid although neither is formed rapidly. The C^{14}-contents of raffinose and amino acid carboxyl did not increase after 26 hours.

Seasonal variations in the rate of formation of some of these constituents were investigated next. The specific activities shown in Table V are an index of the rate of formation of the constituent during the 26-hour metabolic period, assuming little utilization in other processes. Actually sucrose should have been utilized to a considerable extent in 26 hours (Table IV) but in spite of that it was still usually the most active substance isolated.

TABLE IV

PHOTOSYNTHETIC ASSIMILATION* OF $C^{14}O_2$ BY COLORADO SPRUCE LEAVES
FEBRUARY 1956

Constituent	Metabolic period		
	6 hr.	26 hr.	72 hr.
	Specific activity, mμc./mM. of carbon		
Glucose + fructose	553	618	845
Sucrose	1940	1390	830
Raffinose	475	726	675
Hemicellulose	52	117	180
Cellulose	3.5	3.2	4.4
Sucrose–glucose	1940	1404	845
Sucrose–fructose	1920	1383	825
Hemicellulose–glucose	445	—	690
Hemicellulose–xylose	1.9	—	5.7
Pungenin–glucose	23	31	38
Pungenin–aglycone†	2.5	4.1	11.6
Shikimic acid	2.8	5.6	12.4
Amino acid carboxyl	63	87	74

*1.39 μc. of $C^{14}O_2$ assimilated per gram of fresh weight.
†3,4-Dihydroxyacetophenone.

TABLE V

SEASONAL VARIATION IN RATE OF FORMATION OF SOME CONSTITUENTS OF COLORADO SPRUCE TWIGS: PHOTOSYNTHESIS IN $C^{14}O_2$*

Date (1956)	Material examined	mμc./mM. of carbon‡							
		Glucose + fructose	Sucrose	Raffinose	Pungenin	Shikimic acid	Pinitol	Cellulose	Lignin, vanillin
May 2	1955 leaves	294	810	270	3.7	112	0.5	0.7	0.3
May 22	1955 leaves	1218	1500	—	3.7	30	0.4	10	0.9
June 3	1955 leaves	1830	1910	—	1.1	25	—	7.6	0.8
	buds	ND	ND	ND	ND	ND	ND	36.6	1.2
June 11	1955 leaves	2700	2210	—	1.6	65	3.3	0.6	0.4
	New growth†	309	1793	—	—	—	—	209	128
July 24	1955 leaves	1542	1715	—	3.0	18	—	2.4	0.4
	New growth	816	1605	—	75	76	—	6.3	5.1
Aug. 27	1955 leaves	76	1695	—	8.5	14	—	1.7	0.1
	New growth	54	1487	—	15	20	—	2.7	1.7
Oct. 4	1955 leaves	840	1716	—	10	—	—	1.7	0.3
	New growth	726	1365	—	54	51	2.7	4.8	0.5
Nov. 8	1955 leaves	53	450	325	2.6	12	—	0.7	0.9
	New growth	195	1356	984	28	20	2.8	2.6	0.3
Dec. 11	New growth	593	1428	729	8.8	41	0.6	1.4	0.4

*1.34–1.46 μc. of $C^{14}O_2$ assimilated per gram of fresh weight. Metabolic period was 26 hours.
†Succulent new growth 2–4 cm. long, leaves not fully expanded. In later experiments the leaves are fully expanded in new growth.
‡ND = Not determined: samples too small for methods employed. A dash indicates the compound could not be crystallized.

The leaves formed during 1955 showed an increased rate of cellulose synthesis with the advent of mild weather in May. However, on June 11 when the new growth had burst from the vegetative buds the rate of cellulose formation in the old leaves dropped while the formation of both cellulose and lignin in the succulent new growth became very rapid. The rates of formation of pungenin and shikimic acid were roughly parallel, being most rapid in the new growth. Pinitol formation was never very fast but it may have reached a peak in midsummer when pinitol could not be crystallized. The rate of conversion of sucrose to glucose and fructose was quite variable, being highest in the old leaves in spring and summer when new growth was being formed at a high rate.

Discussion

The most striking seasonal change in composition was the disappearance of raffinose from the leaves in the early spring before growth of the vegetative buds was visible to the naked eye. An analogous change has been observed in seeds of *Pinus thunbergii* Parl. where the raffinose stored in the seeds disappears as soon as germination starts (19).

In an investigation of seasonal changes in the composition of white spruce leaves, carried out in Alberta almost 40 years ago (27), it was found that sugars were present in higher concentration in winter than in summer. Suitable methods were not available then for distinguishing between the different sugars. The "maltose" reported by Lewis and Tuttle (27) was calculated from the increase of reducing sugar obtained on subjecting a citric acid hydrolyzate to further hydrolysis with hydrochloric acid. This "maltose" was really a measure of the melibiose portion of raffinose and the glucose released from pungenin. The great decrease in "maltose" observed in warm weather (27) was thus a measure of the decrease in raffinose and pungenin and is in agreement with the results in this paper.

The sucrose is present in an approximately constant concentration throughout the year, the seasonal changes in carbohydrate concentration being due mainly to changes in the concentrations of raffinose and pungenin. It is difficult to say how much raffinose and pungenin contribute to the freezing resistance of spruce leaves. In winter their contribution to the osmotic pressure is about equal to that of sucrose and the hexoses combined. Raffinose and pungenin probably function mainly as stored foods, since they are readily utilized when growth is rapid.

Spruce cuttings collected in winter were useful for the preparative biosynthesis of sucrose-C^{14} and raffinose-C^{14}. In a 6-hour experiment it was found that 65% of the C^{14} fed, as carbon dioxide, was found in sucrose and 8% was found in raffinose. These sugars were readily isolated. The compact nature of spruce twigs makes it feasible to introduce 10–15 g. of plant material (dry weight basis) into a 600 ml. beaker and still have conditions favorable for photosynthesis. The apparatus required (Fig. 1) is much simpler than one recently described for use with algae or tobacco leaves (13).

It is well known that the tracer technique can be used to detect changes in biosynthetic rates of metabolites while their concentration remains practically constant. The specific activity of a metabolite after a given time will depend on (*a*) the amount of C^{14} incorporated into that compound, (*b*) the amount of dilution by the unlabelled compound originally present, and (*c*) the loss of C^{14} in subsequent conversion of the metabolite to other compounds. In contrast to transitory metabolic intermediates, which are labelled rapidly and lose their isotopic carbon readily on further metabolism in $C^{12}O_2$ (7, 8), the compounds studied in this investigation represent storage products, or end products, which are labelled at a relatively slow rate. Consequently the specific activity is an index of the rate of biosynthesis of the compound in question.

Some of the relative rates of biosynthesis, deduced from specific activity measurements, were as expected. It is not surprising that the rates of cellulose and lignin synthesis should be high in the rapidly developing new growth since the deposition of cell wall material is a necessary feature of plant growth. In winter the mature twigs with dormant buds show a very low rate of labelling of cellulose and lignin but, rather unexpectedly, a relatively high rate of C^{14}-incorporation into hemicellulose. The fact that the C^{14} is practically all in the hemicellulose–glucose, with little in the hemicellulose–xylose, suggests that the hemicellulose contains a glucosan free of xylose. It is possible that this glucosan is of the cellulose type and that the block in cellulose synthesis induced by maturation is in the conversion of short-chain glucosans to cellulose. Recent work with sugar beet leaves has shown that simple glucosans are formed from $C^{14}O_2$ and then later converted to cellulose (29).

The natural cessation of growth in a mature plant might be due to the introduction of certain metabolic blocks. The greatly decreased rate of cellulose synthesis, noted in this work, does not necessarily mean that the block in the "mature" winter twigs is actually in cellulose synthesis. For example, a block in synthesis of protein or nucleoprotein might prevent an increase in protoplasm which may be necessary before formation of more cell wall is possible. However further application of the tracer technique should provide information on which reactions are inhibited during maturation, or in dormancy, and thus aid in our understanding of the regulation of growth and differentiation in plants.

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ÉTUDES SUR LE GENRE SOLIDAGO L.

II. LA MISE EN ÉVIDENCE DE LA POLYPLOÏDIE ENDOTAXONIQUE CHEZ S. PURSHII PORTER ET LES TAXA SIMILAIRES[1]

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Résumé

Chez *Solidago Purshii* Porter, il existe deux cytodèmes, l'un diploïde ($2n = 18$) et l'autre tétraploïde ($2n = 36$), qui peuvent être distingués par la longueur des cellules de garde de leurs stomates foliaires. Les plantes diploïdes possèdent des cellules de garde plus petites que les tétraploïdes. Ces deux entités du *S. Purshii* appartiennent donc à ces groupes de cytodèmes où il existe une corrélation positive entre les nombres chromosomiques et les dimensions de certains caractères morphologiques quantitatifs, tels que la longueur des cellules de garde.

Dans les groupes où les courbes de variations des caractères des divers cytodèmes chevauchent peu ou pas, la distribution géographique des divers cytodèmes du complexe peut être beaucoup plus facilement, rapidement et complètement étudiée en mesurant la longueur des cellules de garde de spécimens d'herbier qu'en déterminant les nombres chromosomiques. Les données présentées indiquent que lorsque l'échantillonnage se fait dans les conditions décrites ici, il suffit, en moyenne, de mesurer la longueur de deux cellules de garde d'une feuille médiane par individu, et de répéter ces déterminations sur deux individus par colonie pour arriver à cette fin. Cependant, il est recommandé, par mesure de prudence, d'augmenter ces nombres de quelques unités, et de toujours faire quelques déterminations directes de nombres chromosomiques pour vérifier l'exactitude de cette technique rapide et parfois aussi pour connaître le nombre chromosomique de certains individus extrêmes des divers cytodèmes dont les cellules de garde ont des longueurs moyennes très voisines.

Introduction

Dans un nombre toujours croissant de taxa d'ordre spécifique, la détermination des nombres chromosomiques révèle que plus d'un nombre existent dans des populations bien établies et que ces variations ne peuvent pas être considérées comme accidentelles et éphémères. Les subdivisions de ces taxa peuvent, au point de vue du contraste cytologique qui permet de les distinguer, être désignées par le terme *cytodèmes* (4).

Le degré de différenciation morphologique macroscopique qui existe entre les cytodèmes d'un même groupe peut varier d'un groupe à l'autre. Dans certains cas les caractères contrastants sont nets et on constate alors que même des taxonomistes conservateurs ont considéré les différentes unités en cause comme des espèces ou au moins des sous-espèces ou variétés distinctes. Dans d'autres cas, ces caractères différentiels sont moins accentués et l'histoire nomenclatorielle de ces taxa est souvent constituée par de nombreuses ségrégations et fusions taxonomiques à des niveaux spécifiques ou infraspécifiques. Enfin, il existe aussi des cytodèmes apparentés qui ne se distinguent pas ou ne semblent pas séparables par des caractères morphologiques macroscopiques du genre de ceux que le taxonomiste classique doit normalement employer.

[1]Manuscrit reçu le 5 juin 1958.
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Il est cependant parfois possible de ségréger ces éléments par une analyse biométrique de caractères morphologiques, macroscopiques et quantitatifs, ou encore par des caractères morphologiques et microscopiques qui se présentent sous forme de variations quantitatives ou qualitatives.

Ces trois catégories de groupes de cytodèmes ne sont pas nettement distinctes mais sont réunies par des groupes plus ou moins intermédiaires entre deux des trois niveaux de différenciation morphologique que nous venons de caractériser.

Les cytodèmes du *Solidago Purshii* Porter, dont il est question ici, semblent appartenir à la dernière de ces trois catégories. Il est possible, cependant, qu'une étude fouillée de leurs caractères morphologiques macroscopiques révélera des distinctions que nous n'avons pu mettre à jour jusqu'à présent.

La présente étude démontre que les cytodèmes de certains des groupes qui appartiennent à la troisième catégorie peuvent être ségrégés par le contraste qui existe entre la longueur des cellules de garde de leurs stomates foliaires respectifs, même lorsqu'ils proviennent de localités écologiquement variables, à l'intérieur d'un type d'habitat en voie d'évolution plus ou moins rapide et non synchronisée d'une localité à l'autre.

Matériel et techniques

À l'automne de 1955, quelques plantes de *S. Purshii* ont été récoltées par J. R. Beaudry à St-Adolphe-de-Howard, à Farnham et à Oka, trois localités de la province de Québec. Des déterminations de nombres chromosomiques faites au cours de l'hiver suivant ont établi que les plantes de St-Adolphe-de-Howard et de Farnham avaient 18 chromosomes dans leur complément somatique et celles d'Oka 36. Ces données établissaient donc l'existence de deux cytodèmes, l'un diploïde ($2n = 18$) et l'autre tétraploïde ($2n = 36$), à l'intérieur du taxon *S. Purshii*, puisque le nombre de base du genre *Solidago* est de 9 (2).

L'automne suivant, soit au cours de celui de 1956, de nouveaux échantillons furent prélevés dans huit tourbières du sud de la province, sur une ligne ouest–est couvrant 150 milles de distance, dans le but d'obtenir des données sur l'abondance relative des deux biotypes et en même temps du matériel qui permettrait d'essayer de les séparer l'un de l'autre par des caractères morphologiques, c'est-à-dire sans avoir recours à l'analyse cytologique.

L'examen rapide des caractères macroscopiques des spécimens d'herbier récoltés au cours de ces herborisations indiqua que les représentants de ces deux biotypes, s'ils existaient dans ces échantillons, ne pouvaient probablement pas être séparés les uns des autres sans étude quantitative. On tenta alors de les caractériser en mesurant la longueur des cellules de garde des stomates de la face inférieure d'une feuille située à mi-hauteur le long de la tige. Ce caractère fut choisi parce qu'il est établi qu'il existe souvent une corrélation positive entre le degré de ploïdie et la longueur des cellules de garde (1, 5).

La longueur de 10 cellules de garde par individu, sur un nombre d'individus variant de trois à neuf par localité, fut donc déterminée. En tout, 490 cellules de garde, appartenant à 49 individus provenant de neuf localités différentes, furent mesurées au moyen d'un micromètre oculaire calibré.

Le tableau I résume ces données.* Il est évident que la longueur des cellules de garde des individus d'Oka est nettement supérieure à celle des individus de toutes les autres colonies. L'examen des données originales, sans analyse statistique, indiqua que tous les individus d'Oka appartenaient à la même population et que cette population était bien distincte de celle(s) représentée(s) par les autres échantillons. Les données originales ont cependant été soumises à l'analyse statistique, tout particulièrement pour déterminer: (1) s'il y a homogénéité ou non à l'intérieur de chacun des échantillons étudiés, et (2) si les échantillons autres que celui d'Oka appartiennent à une seule ou à plus d'une population.

TABLEAU I

LONGUEUR MOYENNE EN MICRONS DE 10 STOMATES DE LA FACE INFÉRIEURE D'UNE FEUILLE SITUÉE À MI-HAUTEUR DE LA TIGE DE DIFFÉRENTES PLANTES DE *S. Purshii* PORTER, RÉCOLTÉES DANS DIFFÉRENTES LOCALITÉS DE LA PROVINCE DE QUÉBEC

	Plantes									
	1	2	3	4	5	6	7	8	9	$\bar{X}$
Oka	38.48	40.72	41.07	40.01	40.14	—	—	—	—	40.08
St-Adolphe-de-Howard	34.40	33.40	32.13	32.72	33.10	33.45	—	—	—	33.20
St-Pierre-de-Howick	31.56	32.62	32.62	32.28	32.51	32.26	—	—	—	32.31
Rougemont	33.18	32.99	30.35	32.08	34.34	33.18	32.75	31.48	31.54	32.43
Farnham	33.24	31.89	32.00	32.02	31.45	32.21	32.94	32.54	—	32.29
St-Hubert	33.45	32.64	33.02	—	—	—	—	—	—	33.04
Knowlton	32.56	32.89	32.54	—	—	—	—	—	—	32.66
Coleraine	30.73	32.18	32.00	32.16	—	—	—	—	—	31.77
Lemieux	33.10	31.67	32.37	32.67	34.21	—	—	—	—	32.80

L'analyse du tableau II indique clairement qu'il y a homogénéité à l'intérieur de tous les échantillons, à l'exception de celui de Rougemont qui contient des individus dont les cellules de garde sont nettement différentes de celles des autres individus du même échantillon. L'examen des données originales de cet échantillon de neuf individus indique assez clairement qu'il y en a deux qui s'écartent un peu des sept autres bien que tous n'aient rien en commun avec les individus de l'échantillon d'Oka.

Trois sources de variations contrôlables statistiquement sont présentées dans nos données: (*a*) la variation entre les mesures des stomates d'une même plante; (*b*) la variation entre les moyennes de différentes plantes à la même localité; (*c*) les variations entre les moyennes de localités. L'analyse du tableau III a été faite en utilisant le point de vue "modèle fixe" (3) et la variation totale a été partagée en ses trois composantes. Elle confirme notre impression que la population d'Oka diffère nettement des autres populations quant à la longueur des cellules de garde. Elle indique aussi que lorsqu'on exclut Oka de l'analyse, il existe probablement un peu d'hétérogénéité entre certaines populations.

*Les données originales peuvent être obtenues de J. R. Beaudry.

Tout en faisant l'étude des cellules de garde des plantes de ces échantillons, une nouvelle série de déterminations de nombres chromosomiques sur d'autres plantes provenant d'Oka et sur la progéniture de quelques-unes des plantes des autres échantillons, à l'exception de St-Pierre-de-Howick, a été faite. Le

TABLEAU II

ANALYSE DE LA VARIANCE DE LA LONGUEUR (EN MICRONS) DE 10 DIFFÉRENTS STOMATES DE LA FACE INFÉRIEURE D'UNE FEUILLE SITUÉE À MI-HAUTEUR DE LA TIGE DE DIFFÉRENTES PLANTES DE *S. Purshii*, RÉCOLTÉES DANS DIFFÉRENTES LOCALITÉS DE LA PROVINCE DE QUÉBEC

Localités	Sources de la variation	Sommes de carrés	Degrés de liberté	Moyennes des sommes de carrés	Valeurs de F		
					Calculées	À $P=.05$	À $P=.01$
Oka	Inter-plantes	39.73	4	9.93	1.42	2.57	3.77
	Intra-plantes	314.02	45	6.98			
	Total	353.75	49				
St-Adolphe-de-Howard	Inter-plantes	28.73	5	5.75	1.09	2.38	3.37
	Intra-plantes	285.69	54	5.29			
	Total	314.42	59				
St-Pierre-de-Howick	Inter-plantes	7.93	5	1.59	0.36	2.38	3.37
	Intra-plantes	240.79	54	4.46			
	Total	248.72	59				
Rougemont	Inter-plantes	113.76	8	14.22	3.70*	2.06	2.74
	Intra-plantes	310.78	81	3.84			
	Total	424.54	89				
Farnham	Inter-plantes	24.04	7	3.43	1.01	2.14	2.91
	Intra-plantes	245.37	72	3.41			
	Total	269.41	79				
St-Hubert	Inter-plantes	3.07	2	1.53	0.45	3.35	5.49
	Intra-plantes	91.46	27	3.39			
	Total	94.53	29				
Knowlton	Inter-plantes	0.76	2	.38	0.07	3.35	5.49
	Intra-plantes	141.61	27	5.24			
	Total	142.37	29				
Coleraine	Inter-plantes	14.62	3	4.87	1.14	2.87	4.38
	Intra-plantes	153.57	36	4.27			
	Total	168.19	39				
Lemieux	Inter-plantes	35.50	4	8.88	1.09	2.57	3.77
	Intra-plantes	366.22	45	8.14			
	Total	401.72	49				

*Valeur très significative.

TABLEAU III

ANALYSE DE LA VARIANCE D'ÉCHANTILLONS DE PLANTES DIPLOÏDES ET TÉTRAPLOÏDES DE *S. Purshii*, RÉCOLTÉES DANS DIFFÉRENTES LOCALITÉS DE LA PROVINCE DE QUÉBEC

Sources de la variation	Sommes de carrés	Degrés de liberté	Moyennes des sommes de carrés	Valeurs de F		
				Calculées	À $P=.05$	À $P=.01$
Entre les localités	2600.80	8	325.10	64.68	1.96	2.55
Oka vs. les autres localités	2473.53	1	2473.53	369.18	4.08	7.31
Reste	127.27	7	18.18	2.71	2.25	3.12
Intra-localités	2417.63	481	5.03			
Inter-plantes	268.14	40	6.70	1.38	1.43	1.64
Intra-plantes	2149.49	441	4.87			
Total	5018.43	489				

NOTE: Caractère mesuré: longueur des stomates en microns.

tableau IV montre qu'il y a correspondance constante entre le nombre chromosomique et la longueur des cellules de garde, c'est-à-dire que chez le *S. Purshii*, tel que représenté par ces échantillons, une cellule de garde d'environ 31–34 μ correspond à un nombre diploïde de chromosomes et une autre d'environ 38–41 μ à un nombre tétraploïde. Il est possible, cependant, que l'écart entre les deux valeurs extrêmes pour les plantes tétraploïdes soit supérieur à 3 μ puisque la variation inter-localités n'a pas été estimée pour ces plantes.

TABLEAU IV

NOMBRES CHROMOSOMIQUES SOMATIQUES D'INDIVIDUS DE *S. Purshii* DE DIFFÉRENTES LOCALITÉS DE LA PROVINCE DE QUÉBEC OU DE LA PROGÉNITURE D'INDIVIDUS PROVENANT DE CERTAINES DE CES LOCALITÉS

Localités	Nombre d'individus	Nombres somatiques	
		Individus récoltés dans ces localités	Individus de la progéniture des individus récoltés dans ces localités
Oka	6	36	—
St-Adolphe-de-Howard	11	18	—
St-Pierre-de-Howick*	—	—	—
Rougemont	6 (2)†		18
Farnham	5 (2)		18
St-Hubert	1		18
Knowlton	7 (3)		18
Coleraine	2 (1)		18
Lemieux	5 (2)		18

*Nous n'avons pas encore réussi à déterminer le nombre chromosomique des individus de cette localité.
†Le nombre entre parenthèses indique le nombre de plantes-mères différentes.

Discussion

Plusieurs auteurs ont insisté sur le fait qu'il n'existe pas toujours une corrélation positive entre les nombres chromosomiques et certains caractères morphologiques quantitatifs, tels que la longueur des cellules de garde des stomates, des entités qui constituent une série polyploïde (6). Plusieurs facteurs sont, sans doute, responsables de cette absence de corrélation mais les principaux semblent être les suivants: (*a*) le génotype de l'espèce à l'origine d'une série, tout particulièrement dans le cas des séries à autoploïdie; (*b*) un degré d'identité assez éloigné entre les unités en cause comme conséquence de leur différenciation depuis le moment de leur ségrégation; (*c*) l'introduction d'un génome étranger aux autres éléments de la série par alloploïdie; (*d*) l'hybridation suivie ou non d'introgression et (*e*) l'action du milieu. Ces facteurs doivent pouvoir agir non seulement indépendamment mais aussi en combinaison, et leurs effets peuvent alors être particulièrement accentués.

Si l'alloploïdie agit tel que supposé plus haut, on peut s'attendre à ce que les différences soient plus nettes entre un diploïde et son autoploïde, surtout si ce dernier est d'origine récente. Les deux cytodèmes de *S. Purshii* constituent peut-être un tel cas, mais cela est loin d'être certain.

Les deux individus de l'échantillon de Rougemont qui diffèrent des autres par la longueur de leurs cellules de garde sont peut-être des produits d'introgression entre l'espèce qui domine leur phénotype et une autre espèce inconnue. Nous savons que le cytodème de *S. Purshii* à 18 chromosomes hybride avec le *S. rugosa* Ait., espèce commensale de la première dans la tourbière de Rougemont.

L'action du milieu est assez clairement indiquée chez les spécimens de Coleraine (comté de Mégantic). Ces plantes, qui proviennent d'un sol appalachien pauvre et d'une localité où toute la végétation est couverte par des particules fines, qui s'échappent sous forme de poussière des mines d'amiante de la région et qui nuisent sans doute à la photosynthèse, sont rabougries. C'est probablement pour cela que leurs cellules de garde sont, en général, moins longues que celles des plantes des autres échantillons diploïdes.

Cette étude indique que si les échantillons que nous venons d'analyser sont représentatifs de la population constituée par *S. Purshii*, la distribution des deux cytodèmes peut être facilement et rapidement étudiée en mesurant la longueur des stomates de spécimens d'herbier. Nous ne concluons pas, cependant, que cette technique est applicable à tous les groupes de cytodèmes, puisque, tel que dit plus haut, chez certains de ces groupes, l'action du génotype, de l'alloploïdie, de l'hybridation et celle de l'évolution progressive, combinées à celle du milieu, peuvent faire obstacle à l'existence de la corrélation que nous avons décelée chez *S. Purshii*. Il est évident que chaque groupe doit être soumis à une étude préliminaire pour déterminer si cette technique est applicable ou non.

Afin de tirer des conclusions qui aideront à la planification d'expériences futures, on utilise le "modèle aléatoire" (modèle 2 d'Eisenhart) (3). L'analyse de la variance pour les plantes diploïdes, c'est-à-dire sans les plantes d'Oka, est présentée au tableau V. Les termes de la colonne "Moyennes des sommes de carrés" sont des estimations non biaisées des termes correspondants de la colonne "Espérances mathématiques . . .". Comme les localités, les plantes et les cellules de gardes ont été choisies au hasard, on peut parler de la variance attachée aux localités (inter-localités), aux plantes (inter-plantes) et aux cellules de gardes (inter-mesures ou comme désignées précédemment intra-plantes). Ces trois variances sont dénotées respectivement par σ_L^2, σ_P^2 et σ^2.

TABLEAU V

ANALYSE DE LA VARIANCE D'ÉCHANTILLONS DE PLANTES DIPLOÏDES DE *S. Purshii* RÉCOLTÉES DANS DIFFÉRENTES LOCALITÉS DE LA PROVINCE DE QUÉBEC

Sources de la variation	Sommes de carrés	Degrés de liberté	Moyennes des sommes de carrés	Espérances mathématiques des moyennes des sommes de carrés
Inter-localités	67.11	7	9.59	$\sigma^2 + 10\,\sigma_P^2 + 53.57\,\sigma_L^2$
Inter-plantes	228.41	36	6.34	$\sigma^2 + 10\,\sigma_P^2$
Intra-plantes	1835.48	396	4.63	σ^2
Total	2131.00	439		

NOTE: Caractère mesuré: longueur des stomates en microns.

Donc pour les plantes à 18 chromosomes $\sigma_{(18)}^2 \doteq^* 4.63$; $\sigma_{P(18)}^2 \doteq 0.17$ et $\sigma_{L(18)} \doteq 0.06$. Pour les plantes à 36 chromosomes, on trouve directement à partir des données de l'analyse du tableau II: $\sigma_{(36)}^2 \doteq 6.98$ et $\sigma_{P(36)}^2 \doteq 0.30$. On ne peut estimer $\sigma_{L(36)}^2$ car on a une seule localité. On supposera que $\sigma_{L(36)}^2$ est aussi estimé par 0.06.

Comme la variance de la différence entre les moyennes pour deux localités, l'une où les plantes sont diploïdes et l'autre où elles sont tétraploïdes, est estimée par: $(\sigma_{(18)}^2/m \times n) + (\sigma_{P(18)}^2/m) + (\sigma_{L(18)}^2/1) + (\sigma_{(36)}^2/m \times n) + (\sigma_{P(36)}^2/m) + (\sigma_{L(36)}^2/1)$, où il y a m mesures par plante et n plantes par localité, on voit (par tâtonnement) que pour $m = n = 2$, l'estimation de cette variance est: $(4.63/2 \times 2) + (0.17/2) + (0.06/1) + (6.98/2 \times 2) + (0.30/2) + (0.06/1) = 3.26$.

Donc l'écart standard est de $\sqrt{3.26}$ ou 1.8. Si le problème consiste à distinguer entre les plantes à 18 chromosomes et les plantes à 36 chromosomes, il s'agit de déceler une différence dont la valeur est plus grande que 6 μ, et on voit qu'un échantillon de deux plantes par localité et de deux cellules de garde par plante suffit. Cependant, comme certains échantillons peuvent être plus variables que ceux de la présente étude, il est recommandable d'augmenter ces nombres de quelques unités. Cette précaution est d'autant plus justifiable que la variation inter-localités des plantes à 36 chromosomes n'a pas été estimée.

Il nous reste maintenant à appliquer cette technique dans les conditions qui existent dans les herbiers. C'est ce que nous faisons maintenant et que nous rapporterons dans un autre article. Disons dès maintenant que ces conditions diffèrent, en général, de celles décrites plus haut surtout par le fait que chaque localité est rarement représentée par plus d'un individu. À cause de cela il est donc généralement impossible d'estimer la variation entre les plantes d'une même localité, la variation inter-localités étant confondue avec la variation inter-plantes. Pour tenter d'obvier au moins partiellement à ces inconvénients, il semble recommandable d'augmenter substantiellement le nombre de mensurations par individu.

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*≐ signifie *est estimé par.*

Summary

In *Solidago Purshii* Porter, there are two cytodemes, one diploid ($2n = 18$) and the other tetraploid ($2n = 36$), which can be distinguished on the basis of the length of the guard cells of their leaf stomata. The diploid plants possess guard cells which are smaller than those of the tetraploid. These two entities of *S. Purshii* thus belong to the groups of cytodemes in which there exists a positive correlation between the chromosome numbers and the dimensions of certain quantitative morphological characters, such as the length of the guard cells.

In groups in which the variation curves of the characters of the different cytodemes overlap little or not, the geographical distribution of the different cytodemes of the complex may be studied much more easily, rapidly, and comprehensively by measuring the length of the guard cells of herbarium specimens than by making chromosome number determinations. The data presented indicate that when sampling is made under the conditions described in this paper, determinations of the length of two guard cells of stomata of one mid-leaf per individual, made on two individuals per colony, is satisfactory for that purpose, on the average. However, it is recommended, as a safeguard, to increase these figures by a few units, and to make always a few direct chromosome number determinations to check the accuracy of this rapid technique. These direct determinations may also be necessary to know the chromosome numbers of certain extreme individuals of the different cytodemes which have guard cells that differ little in length.

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THE DISTRIBUTION OF SAVANNA SPECIES IN RELATION TO LIGHT INTENSITY[1]

J. R. BRAY

Abstract

The distribution of savanna species in relation to light intensity was investigated during ecologic study of the prairie–forest border in Wisconsin. Four thousand intensity measurements were made in 17 stands, of which 915 were taken over sampled quadrats. Measurements were made on clear days during the midday period in July and August with a Weston photometer, and readings from different stands were considered comparable. Light amplitudes were determined by calculating the percentage of a species in the quadrats of each intensity size class. These classes were based on a log 10 scale after schemes of linear and geometric progression indicated curvilinear distributions would most likely result from a log scale. Forty-seven species distributions were calculated, 43 of which showed normal patterning with distinct modes.

The log 10 scale was used to show distribution of light intensity in 16 stands along a gradient from prairie to terminal forest.

Light distributions of species on two soil moisture levels showed a highly significant tendency for the lower light amplitude to occur on the drier moisture level. This tendency operated for species at all levels of the soil moisture gradient.

Possible mechanisms for the species–light distributions were discussed in relation to growth–light curves and to conditions of soil moisture and evaporation which are correlated with or controlled by light intensity.

Introduction

During ecologic study of the prairie–forest border in Wisconsin, measurements of light intensity were taken over an extensive series of sampled quadrats. The following analysis presents the results of these measurements and an interpretation of their ecologic significance.

Methods

The study area included the region of prairie occurrence mapped by Curtis (6) in Wisconsin and a strip in Minnesota along the Mississippi River from Winona south to Brownsville. Within this area, light measurements were made in prairie, savanna, and forest stands. No forested stand was sampled in which there was evidence of past cutting. Readings over sampled understory quadrats were taken only in stands which were in original condition or in which recovery from past grazing was nearly complete.

The study was made during the months of July and August in the years 1951 to 1954.

Measurements of light intensity were taken with a Weston photometer calibrated in three scales: 0–100 ft-c.; 0–1000 ft-c.; and 0–10,000 ft-c. The instrument was checked by the Weston Company once during the course of the study. At each intensity reading, the paddle containing the photoelectric cell was held by hand horizontal to the surface at a position 3 ft.

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TABLE I

SAVANNA LIGHT INTENSITY DISTRIBUTION PER 1,000 UNIT INTERVALS

	% distribution by the designated intensity intervals (ft-c.)											
	0–999	1000–1999	2000–2999	3000–3999	4000–4999	5000–5999	6000–6999	7000–7999	8000–8999	9000–9999	10,000+	Total No. values
Distribution, all values	37.5	15.4	5.4	4.4	2.3	2.3	2.0	1.0	1.1	1.1	27.6	2,830
Albion oak-opening	18.0	10.9	4.3	4.5	2.7	2.8	2.0	1.2	1.5	1.7	50.4	1,000
Shade from lone trees	25.7	34.3	10.5	5.7	3.8	5.7	3.8	0.0	3.8	6.6	—	105
Ant (closing oak-opening)	48.2	21.7	7.6	4.8	2.0	2.0	2.6	1.4	0.9	0.6	8.2	1,000
Trempealeau (closing canopy)	67.8	11.6	4.5	0.9	2.7	1.8	0.9	0.9	1.8	0.9	6.2	112

above the ground. If there was a fluctuating dial position during this reading, the median position was chosen. This fluctuation resulted from wind movement in the canopy, which caused variations of as high as 3000–4000 ft-c. At the higher light intensities, the paddle was never exposed for longer than two seconds to prevent desensitization of the cell.

Measurements were taken at random or contiguous stations, and over 1 × 1 meter quadrats which were scattered at random. Within these quadrats, presence of herb and shrub species and of tree reproduction was recorded. A single light measurement or a composite of four measurements was made at each station or quadrat.

Intensity readings were made only on clear days from 9 hours to 15 hours, with the vast majority of readings taken between 10–14 hours, a period in which intensity within the sample area is at least 10,000 ft-c., but below the 12,000 ft-c. maximum recorded for high mountain altitudes. Variation in intensity during this period is mainly dependent on optical air mass (m) which varies with the sun's zenith distance. For a latitude of 40° N, m varies from 1.18 to 1.06 for July 24 and from 1.30 to 1.15 for August 28, during the 10–14-hour period. In both cases, this variation is less than 10% of the higher value (see Tables 137 and 170, List (13)). The occurrence of a plateau in light intensity during the midday period has been frequently demonstrated (Briggs and Shantz (4), Thomas and Hill (16), Byram and Jemison (5)). It is assumed that the distribution of intensity values in a stand as a whole was the same throughout the midday period and that a series of readings made at any time during the period would show approximately similar patterns of species distribution.

The level of intensity in full sunlight was repeatedly checked during a survey to insure that the unshaded intensity remained at 10,000+ ft-c. This checking was easily accomplished since each of the stands had areas of open canopy or was of small extent and could be checked at the forest edge.

Since intensity readings were taken only during the maximum intensity plateau and on clear days, it was considered that values from different stands were directly comparable. Information available from the field study included (1) the midday range of values and the average values within a stand; (2) the range in intensity conditions, during the midday period, under which a species occurred; (3) the degree to which a species predominated at a given intensity level during that period.

Seventeen stands were studied, including 10 which contained undisturbed understory vegetation. Four thousand readings were taken, of which 915 were made over sampled quadrats.

Results and Discussion

Selected distributions of light intensity values per stand are shown in Table 1. Line 2 demonstrates the intensity range under Albion bur oak opening, a stand in which each tree was open-grown and in which few canopies were in contact. The low density of trees per acre in Albion is shown by

the occurrence of most of the values in the 10,000+ ft-c. interval. Line 3 demonstrates the substantial shading action of bur oak crowns of lone trees surrounded by prairie with 60.0% of the values below the 2000 ft-c. interval. The last two rows in the table show stages in the closing of oak-opening canopies, with a decrease, in row 5, to 6.2% in the maximum intensity class and an increase to 67.8% in the class below 1000 ft-c.

It is apparent from Table I that values immediately beneath the daytime maximum of 10,000+ ft-c. are poorly represented in savanna (and forest) stands. Line 1, which represents the distribution of all savanna values in the study, shows, for example, one half of the intensity range (4000–9000 ft-c.) represented by only 9.8% of the readings. This shortage of intermediate values is the result of the rapid diminution in direct light which occurs if any degree of shading by leaf and branch canopy is present. The occurrence of direct light was rare or absent in closed canopy stands, but in savanna, most of the readings were median estimates of the fluctuations which occurred under thin canopy covers through which filtered, with even the slightest wind movement, patterns of shifting sun flecks. A second cause of decreased intensity readings, independent of direct canopy shading, is a decreasing diffuse light (sky light) with an increasing density of trees per acre.

Patterns of Species Distribution

Since light intensity values do not show a linear distribution, species distribution in relation to light intensity must be based on the per cent occurrence of a species within a given intensity size class. The data from all stands with understory samples (total 10 stands) were combined to determine species distributions. If a species did not occur in a stand, a failure perhaps related to geographic or historic factors, the light intensity data from that stand was not included in determining the per cent values.

The limits of the light intensity class intervals were based on the number of available values per interval and on the nature of the resultant species distribution patterns. The first scheme investigated was a linear progression as follows; 0–999, 1000–1999; 2000–2999;... 10,000+. This scheme showed that most species distributions had junctures of rapid increase or decrease which separated a plateau of relatively unchanging occurrence. The paucity of values in the intermediate size classes (4000–9000 ft-c.) necessitated combining these classes in some cases so that within this range it was not possible to determine distribution trends. Nevertheless, most species patterns showed a change in relative occurrence between the 0–999 and the 1000–1999 levels and between the 9000–9999 and the 10,000+ ft-c. levels. Between these junctures an increase in intensity made little or no consistent change in per cent occurrence.

The second scheme was based on a geometric progression as follows: 0–187; 188–374; 375–749; 750–1499; 1500–2999; 3000–5999; 6000–12,000. Species distributions according to the geometric progression showed patterns which could be consolidated to the following scheme: 0–187; 188–749; 750–5999; 6000–12,000. If the linear and geometric schemes are considered jointly,

the following distribution of class intervals can be summarized: 0–187; 188 to 750–1000; 750–1000 to 6000–10,000; above 6000–10,000. This distribution suggests a log 10 scheme of 0–99; 100–999; 1000–9999; 10,000+. Species distributions in Table II are based on this scheme and are calculated by the method noted above. *Amorpha canescens*, for example, in the 10,000+ ft-c. class occurred in 166.75 of the 223.75 eligible quadrats in the class interval for a per cent occurrence of 74.5. Values for quadrat occurrence appear in quarters since, in three of the studies, light measurements were taken over each quarter of a quadrat. Nomenclature in Table II and throughout the text is after Gleason (9).

The distribution patterns in Table II demonstrate an ordered occurrence in relation to intensity level for most of the included species. All species in the study which occurred with at least 10% frequency in one of the class intervals are represented in the table. The curvilinear trend of 43 out of 47 of these patterns indicates the utility of the chosen class intervals.

The order of species in Table II is by a weighted index shown in column 5 which is based on increasing occurrence toward the lower intensity intervals. Species toward the beginning of the table are found predominantly in prairie and other open communities, whereas species to the center of the table are the dominant members in savanna and open woodland. A few of the species which peak toward the lower intensity intervals are more prevalent in closed canopy forests, as *Solidago ulmifolia*, *Smilacina racemosa*, and *Aralia nudicaulis*.

Patterns of Stand Distribution

The intensity divisions of Table II are useful in showing the distribution of light within savanna stands and within stands with varying degrees of closed canopy. Table III shows 16 stands along a gradient from prairie to terminal forest. The distribution of values varies from prairie, in which all light is at the 10,000+ ft-c. maximum during the midday period, to Denmark Beech, a closed and densely canopied maple–beech–hemlock forest, in which 95% of the values are beneath 100 ft-c. Each size class in the table is represented by peak values in per cent occurrence and the intensity intervals approximate a scale which demonstrates an even transition along the open to closed forest gradient. The occurrence of 2 of the 20 summary readings in Denmark Beech below 10 ft-c. suggests an additional division of 0.0–9.9 ft-c. might be utilized. The quadrats with readings below 10 ft-c. were almost bare of plants. These readings were taken under the additional canopy cover of low understory trees and saplings.

Light Intensity – Soil Moisture Relationships

It was evident from field observation that the ecologic light intensity amplitude of a species might be related to its soil moisture amplitude. Plants which reached peak importance towards the mesic and moist end of the prairie continuum of Curtis (6) were often noted beneath the canopies of trees which were scattered through an area whose species complement in the open was

TABLE II

SPECIES DISTRIBUTION BY LIGHT INTENSITY INTERVALS

		0–99	100–999	1000–9999	10,000+	WLP*
Muhlenbergia cuspidata		—	0.0	6.8	36.4	3.84
Petalostemum purpureum		0.0	0.7	6.5	43.2	3.84
Andropogon scoparius		0.0	4.0	11.9	51.1	3.70
Andropogon scoparius	M	0.0	5.2	11.2	17.0	3.35
Andropogon scoparius	M–D	—	3.5	12.0	53.0	3.72
Kuhnia eupatorioides		—	1.0	11.7	29.8	3.68
Aster sericeus		—	3.2	9.8	34.2	3.66
Bouteloua curtipendula		0.0	2.6	16.4	41.6	3.64
Bouteloua curtipendula	M	—	1.4	3.5	13.3	3.65
Bouteloua curtipendula	M–D	—	3.0	18.2	43.2	3.62
Solidago rigida		—	3.3	1.2	13.4	3.56
Stipa spartea		—	14.8	30.0	53.0	3.39
Helianthus laetiflorus		—	13.8	26.2	45.9	3.37
Panicum leibergii		—	10.1	29.8	38.0	3.36
Petalostemum candidum		0.0	5.6	13.4	16.3	3.30
Amorpha canescens		1.7	24.8	54.9	74.5	3.30
Amorpha canescens	M	2.5	10.5	12.3	35.7	3.43
Amorpha canescens	M–D	—	30.5	60.8	76.4	3.27
Andropogon gerardi		0.0	18.3	29.5	44.7	3.28
Andropogon gerardi	M	0.0	16.0	26.0	57.1	3.41
Andropogon gerardi	M–D	—	19.2	30.0	44.1	3.27
Aster ericoides		—	11.7	19.2	23.2	3.21
*Comandra richardsiana**		0.0	6.7	26.3	15.5	3.18
Lithospermum canescens		0.0	6.8	15.9	13.5	3.18
Lithospermum canescens	M	—	8.5	23.0	17.1	3.18
Lithospermum canescens	M–D	—	6.0	14.5	13.3	3.22
*Apocynum androsaemifolium**		0.0	21.2	17.1	28.0	3.10
Apocynum androsaemifolium	M	—	11.0	8.8	36.7	3.45
Apocynum androsaemifolium	M–D	—	24.4	18.0	27.7	3.05
Antennaria sp.		0.0	7.6	10.6	9.9	3.08
*Smilacina stellata**		—	11.3	22.0	14.1	3.06
Coreopsis palmata		0.0	37.0	44.4	38.6	3.01
Coreopsis palmata	M	—	11.0	23.3	35.7	3.35
Coreopsis palmata	M–D	—	47.8	47.5	38.8	2.93
*Elymus canadensis**		—	26.5	46.5	24.5	2.98
Euphorbia corollata		13.6	31.7	56.7	52.3	2.96
Euphorbia corollata	M	0.0	21.1	20.7	66.0	3.42
Euphorbia corollata	M–D	—	36.6	64.5	51.6	3.10
Lespedeza capitata		0.0	6.4	23.6	4.4	2.94
Rhus glabra		—	4.9	13.3	3.2	2.92
Anemone cylindrica		0.0	8.9	15.1	6.2	2.91
Rosa suffulta		0.0	28.6	29.7	21.6	2.91
Rosa suffulta	M	—	21.0	28.8	78.6	3.45
Rosa suffulta	M–D	—	31.6	29.8	18.8	2.84
*Corylus americana**		—	41.3	24.8	31.4	2.90
Quercus macrocarpa		—	7.6	11.1	3.8	2.83
*Celastrus scandens**		2.8	25.8	31.9	14.8	2.78
Celastrus scandens	M	—	23.2	21.9	26.2	3.04
Celastrus scandens	M–D	—	27.2	34.0	13.8	2.82
*Monarda fistulosa**		0.0	30.1	39.6	10.1	2.75
Monarda fistulosa	M	—	24.9	34.2	42.9	3.18
Monarda fistulosa	M–D	—	32.3	40.5	7.6	2.69
Phlox pilosa		—	24.3	17.9	10.6	2.74
Aster azureus		—	18.5	14.2	5.7	2.67
Aster azureus	M	—	17.5	35.0	40.0	3.24
Aster azureus	M–D	—	19.1	8.1	2.8	2.46
*Ratibida pinnata**		—	17.0	15.2	3.8	2.63
*Fragaria virginiana**		1.7	20.3	21.2	4.8	2.61
Fragaria virginiana	M	—	6.6	12.1	8.5	3.07
Fragaria virginiana	M–D	—	27.2	23.6	4.4	2.59

TABLE II—*Continued*

SPECIES DISTRIBUTION BY LIGHT INTENSITY INTERVALS

		0–99	100–999	1000–9999	10,000+	WLP*
Helianthus strumosus		—	45.7	27.2	9.9	2.57
Helianthus strumosus	M	—	33.8	40.3	40.0	3.05
Helianthus strumosus	M–D	—	51.3	25.4	8.7	2.50
*Ceanothus americanus**		3.4	13.2	11.5	5.3	2.56
Heliopsis helianthoides		25.4	37.5	36.4	21.3	2.44
Heliopsis helianthoides	M	0.0	18.3	19.8	21.3	3.05
Heliopsis helianthoides	M–D	—	63.9	57.0	21.4	2.70
*Aster laevis**		—	27.0	19.5	0.0	2.42
Solidago ulmifolia		—	27.1	9.8	2.4	2.37
Solidago ulmifolia	M	—	48.3	42.1	40.0	2.94
Solidago ulmifolia	M–D	—	17.8	4.5	0.0	2.20
Pteridium aquilinum		1.9	12.2	9.9	0.0	2.33
Amphicarpa bracteata		30.5	67.3	48.3	14.3	2.29
Amphicarpa bracteata	M	22.5	58.2	61.2	34.0	2.84
Amphicarpa bracteata	M–D	—	71.9	44.8	12.6	2.54
*Galium boreale**		37.3	41.1	44.5	14.3	2.26
Galium boreale	M	20.0	16.4	20.7	10.6	2.88
Galium boreale	M–D	—	52.5	49.7	14.5	2.67
Carex pensylvanica		15.4	31.4	16.1	4.4	2.14
Cornus racemosa		33.9	28.1	15.7	2.8	1.84
Cornus racemosa	M	32.5	18.8	12.3	19.0	3.00
Cornus racemosa	M–D	—	31.8	16.1	2.0	2.40
Smilacina racemosa		21.6	9.4	11.4	0.7	1.80
Geranium maculatum		28.8	17.8	4.1	1.6	1.59
Geranium maculatum	M	37.5	26.8	12.1	4.2	2.48
Geranium maculatum	M–D	—	13.0	2.3	1.5	2.31
Aralia nudicaulis		39.0	19.2	5.8	0.0	1.48

*The first four columns indicate per cent frequency within the quadrats of each intensity class. Column 5 gives the weighted position of the species within the first four columns. Intensity intervals in foot candles. M indicates mesic savanna; M–D indicates mesic–dry savanna. Species marked with asterisk were often noted in shaded areas of drier prairie than they normally occupied.

TABLE III

LIGHT INTENSITY DISTRIBUTION PER STAND*

	Intensity interval			
	0–99	100–999	1,000–9,999	10,000+
Stand				
Any prairie	0.0	0.0	0.0	100.0
Nashota	0.0	4.0	15.0	81.0
Scuppernong	0.0	8.0	10.0	82.0
Albion	0.0	18.0	31.6	50.4
Heger City	0.0	27.0	41.0	32.0
Garwin Heights	0.0	25.0	47.0	28.0
Basco	0.0	34.2	61.4	4.4
Columbia Golf	0.0	40.0	55.0	5.0
Ant Woods	0.1	48.1	43.6	8.2
Trempealeau II	3.0	66.0	25.0	6.0
Fountain City	13.0	56.0	25.0	6.0
Du Charme	15.0	50.0	35.0	0.0
Brownsville	10.0	77.0	12.0	1.0
Trempealeau I	26.0	57.0	17.0	0.0
Sherwood Point	30.0	55.0	15.0	0.0
Denmark Beech	95.0	5.0	0.0	0.0

*Values in body of tables are the per cent occurrence of light measurements in the designated intensity intervals.

characteristic of dry prairie. The terms "moist", "mesic", and "dry" were used by Curtis to designate the over-all soil moisture characteristics of a gradient which was constructed by a consideration of vegetational relationships. Correlated with species distributions in prairie was a soil water retaining capacity from low (dry) through medium (mesic) to high (moist). Other characteristics associated with the dry end of the gradient are shallow soils, high specific gravity, and good surface and subsurface drainage, while the moist end of the gradient shows deeper soils, poor drainage, and a higher organic matter content and, therefore, a lower specific gravity.

In order to examine the relationship between light and moisture amplitudes, the position of stands along the prairie continuum was determined to locate a possible differential behavior of species in relation to light at varying soil moisture levels. A prairie continuum index was calculated for each of the 10 stands included in Table II by weighting the per cent frequencies of species in the stand by adaptation values determined by weighted location in Table I of Curtis (6). Adaptation values were calculated by multiplying the per cent presence of the species in Table I of Curtis by the rank number of the column heading, and dividing the sum of these weightings by the sum of the per cent presence values. *Amorpha canescens*, for example, was assigned an adaptation value of 4.97, indicating it reached maximum presence toward the right or "dry" end of the continuum. In a calculation of the continuum position of each stand in the present study, only quadrat samples in the areas of the stand which were unshaded during the midday period were utilized. An attempt was made, thereby, to locate the prairie continuum position of each stand independent of the direct shading influence of scattered tree canopies.

The result of the computation of prairie continuum indexes showed a range in index value from 3.94 to 4.96, a range which extended, in general, from mesic to mesic–dry communities. This range was halved, with three stands occurring in the lower, more mesic half, and with seven stands in the upper, mesic–dry half. Light amplitudes on a log 10 scale were then determined, for each of the moisture levels, by the techniques of per cent species occurrence which were previously outlined. Twenty species were represented by sufficient values to give per cent occurrence of 10 or higher in at least one interval on both moisture levels. These patterns are represented in Table II by two additional rows beneath the previously outlined light amplitudes.

An examination of the light amplitudes of Table II, on each moisture level, clearly reveals a shift, in the dryer of the two soil moisture classes, to the lower light intensity classes. This shift is demonstrated by the index based on the per cent occurrence of a species weighted by the number of the column heading, as shown in column 5. The higher the magnitude of the index value, the more nearly the light intensity amplitude is towards the higher intensity classes. In 18 of the 20 patterns in Table II, the lower light index value occurs on the less mesic soil moisture level. This shift could only be expected by chance with a probability of $< .01$. The light and

soil moisture amplitudes of a species are, therefore, highly significantly related, and, as a species occurs in communities of decreasing soil moisture (in the open), it can be expected to occur increasingly toward the lower light intensity classes. This shift toward lower intensities can be found for species at any position on the prairie continuum. From this relationship it follows that every prairie species may benefit by various degrees of shading in extending its ecologic amplitude to areas beneath the canopies of trees in stands in which the species would otherwise not be able to successfully compete. For any one geographic area, however, the species peaking towards the extreme xeric prairie stands for the area will not have conditions of a greater dryness into which they can migrate by occurring in areas beneath tree canopies. These conditions, for the Wisconsin xeric species, as *Andropogon scoparius*, *Bouteloua hirsuta*, and *Muhlenbergia cuspidata*, would be found in the dryer grasslands to the west of Wisconsin. If there were trees in these areas which were growing in xeric prairie, the species which occur beneath their canopies might be expected to include the species mentioned above.

The survival of pine seedlings in forests in which the light intensity was below the apparent minimum for the species was reported by Korstian and Coile (10) in trenched plots in which the seedlings were protected from tree root competition. Results of similar studies have been reviewed by Shirley (15). It is possible that there may be an increase in soil moisture beneath the canopies of savanna trees, which permits a lowering of the light intensity minimum for understory species which is similar to that found in tree seedlings. During the dryest part of the midwestern rain–drought cycle, as in the 1930's, however, the use of soil moisture by tree roots was so efficient that examples were noted of plants which were missing from the nearly bare ground beneath the canopy, but which were present in lawns away from the area of tree root competition.

Possible Mechanisms of the Light Intensity Distributions

Possible mechanisms underlying the curvilinear distributions in Table II include growth–light relationships and the influence of soil moisture and evaporation factors. Available regressions of growth on light intensity indicate, as noted by Shirley (14, 15), Kramer and Decker (12), Kramer and Clark (11), Barney (1), and Decker (7), less change between 1000–2000 ft-c. and full sunlight than between darkness and 1000 ft-c. Blackman and Wilson (2) have shown growth rates which have a curvilinear distribution in relation to the logarithm of light intensity and have contended that effects of shading on net assimilation are similar for a large number of species. They suggest that neither rate of assimilation nor level of compensation point can account for the relative ability of species to withstand shading, and that the varying relationships between growth and light intensity can be attributed, in part, to changes in leaf area ratio. They emphasize that species cannot, therefore, be grouped into "sun" and "shade" plants. A differential ecologic distribution of plants in relation to intensity seems possible, however, if there is a difference in compensation point or in the slope of the assimilation

rate which is sufficient to give a species at a given intensity level a competitive advantage over another species. Such differences are present in the graphs and tables of Blackman and Wilson and in the other noted studies, if results are placed on a log scale. Experimentation on growth–light relationships of native plants (instead of the crop plants usually used) would help analysis of the relative importance of the growth–light curve in determining natural distribution.

The correlation between soil moisture and light amplitude, and the patterns of plateau and juncture in the linear and geometric progression schemes previously noted, suggest the distributions in Table II may be concomitantly influenced by soil moisture and evaporation factors. The junctures in Table II occur during stages in the closing of the forest canopy at which there is the possibility of a greater degree of microclimatic change than in intervening stages. Thus, the first major difference in species composition in the prairie-to-forest transition occurs at the point of initial shading of prairie at the 9999–10,000 ft-c. juncture. Possible decreased soil temperatures directly beneath tree canopies and a subsequent decreased evaporation apparently favor species whose moisture amplitude is increasingly toward the moist prairie continuum intervals. It was noted, from field observation, that there was an occurrence beneath tree canopies of the more aggressive and mobile plants of the moister prairie intervals. Species often observed in shaded areas of drier prairies are marked with an asterisk in Table II. *Salix humilis*, *Veronicastrum virginicum*, and *Viola cucullata* were the most frequent species in this category although sufficient measurements were not available to allow their inclusion in Table II.

It is possible from the large number of species which reach peak compositional importance in savanna that at least a few species may have evolved under the intermediate light intensity classes of savanna. There are, however, no species which are limited to savanna and which do not occur in either forest or prairie (Bray (3)).

The next major shift in species occurrence in relation to light is around 750–1500 ft-c. No microclimatic data other than light are available from the present study but it is suggested that within this juncture there may be a considerable altera tion in the climate of the stand as a whole. At this point, the stand is no longer a savanna, but approaches or has reached a forest in which every tree canopy touches the canopy of at least one neighboring tree. There is usually a greatly increased shrub and sapling growth which augments the canopy crowns and trunks in decreasing wind movement. Geiger (8) refers to a closing of plant cover as a "joining of hands" and notes this closing results in substantial microclimatic change. Decreases in such factors as insolation, temperature, wind movement, and eddy currents may, therefore, be sufficiently great at this juncture to cause relatively sharper decreases in evaporation and increases in soil moisture than occurred within the preceding savanna community. At the 750–1500 ft-c. juncture there

is a competitive situation more favorable to plants of the moist prairie continuum (for example, *Geranium maculatum* and *Viola cucullata*), but, in many cases, the compensation point of prairie species may be reached before 1000 ft-c. (ca. 0.1 full sunlight).

The next major shift in species composition in Table II, at the 75–150 ft-c. level, was not sufficiently encountered in the present study to analyze.

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THE EFFECT OF PRATYLENCHUS PENETRANS ON A CLONE OF FRAGARIA VESCA[1]

J. L. TOWNSHEND[2]

Abstract

The effect of *Pratylenchus penetrans* (Cobb, 1917) Sher & Allen, 1953 on a clone of *Fragaria vesca* was investigated. In the presence of small numbers of *P. penetrans*, the net increase in the number of petioles, petiole length, and fresh weight was significantly less than that of the controls. It would appear by analogy that *P. penetrans* may well be an important primary parasite in the strawberry root-rot complex.

Introduction

Hildebrand (2) frequently found nematodes as well as a number of potentially pathogenic fungi in the roots of the wild strawberry *Fragaria virginiana* grown in soil from strawberry plantings infected with root rot. Hildebrand and West (3) later identified the nematode as *Pratylenchus pratensis* (de Man, 1880). Goheen and Braun (1) found *Pratylenchus penetrans* (Cobb, 1917) Sher & Allen, 1953 in the roots of *F. virginiana* from woodlands in Maryland, U.S.A. In Holland, Klinkenberg (4) demonstrated the pathogenicity of *P. penetrans* on another species of wild strawberry, *F. vesca alpina*. Evidence is presented here that *P. penetrans* is also parasitic on a clone of *F. vesca*.

Materials and Methods

Nematode-free plants of a clone of *F. vesca* were obtained by rooting 20 uniform runner plants in sterile soil in 2-in. pots.

Pratylenchus penetrans was reared on vetch. Soil and strawberry roots from an infected 2-year-old Premier planting were placed in deep flats and planted to vetch. After one month the vetch was dug up, the roots removed, and thoroughly washed to remove saprophytic and ectoparasitic nematodes. The infected roots were spread out in a mat on a mixture of one-half steam-sterilized compost and one-half sand in deep flats. The mat was covered with the soil mixture and planted to vetch. This procedure was repeated monthly until the only parasitic nematode to emerge from the roots was *P. penetrans*. To obtain nematode inoculum, the infected vetch roots were well washed, cut into short lengths, and uniformly mixed. The inoculum was divided into 15 equal parts by weight. Five parts were used to determine the initial level of the nematode population by means of Young's technique (5). The remaining 10 parts were incorporated around the roots of 10 *F. vesca* plants which were then planted in sterile compost in 5-in. pots. A control series of 10 plants was prepared in the same manner except that the vetch roots used contained no nematodes. The 20 plants were randomized in a single block in a growth room at 65° F.

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The number of petioles, the total length of the petioles, and the total weight of each plant were recorded before planting and at the end of the experiment 5 weeks later at which time the nematode population in the roots was also determined by means of Young's technique. The results are recorded in Table I.

Results

The data in Table I show that the average increases in the number of petioles, in the total length of the petioles, and in the total weight of the control plants were respectively two, three, and four times as great as those of the inoculated plants. The average number of *P. penetrans* within the roots of the inoculated plants had increased eight fold. Within the inoculated series the increase in total plant weight was inversely correlated with the final nematode population within the roots. This relationship was significant at the 1% level ($r = -0.858$).

TABLE I

THE EFFECT OF *Pratylenchus penetrans* ON THE GROWTH OF A CLONE OF *Fragaria vesca*

	Growth of strawberry (average net increase per plant)			*Pratylenchus penetrans*	
Treatment	Number of petioles	Petiole length, cm.	Fresh weight, g.	Initial inoculum (per pot)	Final recovery (per root system)
Inoculated	3.9	13.7	2.3	150	1248
Control	7.3	38.9	8.9	0	0
L.S.D. .01	2.9	22.0	5.0		

The top growth in the inoculated series of plants was noticeably retarded after 12 days although there was no chlorosis. At the end of 5 weeks three of the control plants, but none of the inoculated ones, were producing runner plants. Discrete, dark-brown lesions from which *P. penetrans* could almost always be dissected were commonly observed on the roots of inoculated plants but not on the roots of control plants, which were healthy and normal in appearance. The adventitious, lateral, and feeder roots of the more severely stunted plants were greatly reduced and badly discolored (Fig. 1).

Discussion

Pratylenchus penetrans was able to invade and multiply rapidly in the roots of a clone of *F. vesca*. The development of discrete, dark-brown lesions followed the invasion of this endoparasite. Discoloration of the entire root system was observed on the most severely affected plants. The growth rate of the plants in the inoculated series was inversely correlated with the final nematode population extracted from the roots. Thus it would appear that *P. penetrans* is an important primary parasite in the strawberry root-rot

FIG. 1. The effect of *P. penetrans* on a clone of *Fragaria vesca*. The infected plant on the left; the control on the right.

complex. However, it should be stressed that fungi and bacteria undoubtedly were associated with the nematode in these studies and the role of this microflora in the development of the root lesion has not yet been established. The microflora associated with the nematode inoculum may be involved in the further breakdown of lesions initiated by *P. penetrans*.

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GROWTH AND MORPHOGENESIS IN THE CANADIAN FOREST SPECIES

III. THE TIME SCALE OF MORPHOGENESIS AT THE STEM APEX OF PINUS RESINOSA AIT.[1]

G. H. Duff and Norah J. Nolan

Abstract

The main shoot apex of *P. resinosa* is found to comprise four groups of meristematic cells constituting four generative centers by which the parts of the winter terminal bud are laid down. These are the superficial initiating cells, the group or zone of subapical mother cells, the zone of pith mother cells, and the flanking cells.

The superficial primordia of the terminal winter bud pass the winter as secondary lateral budlets on the flanks of the main bud axis. Those of the leaf-bearing short shoots do not normally differentiate leaves until the following spring. Neither do those which develop into lateral long shoots. The fertile budlets, on the contrary, produce cones in the late summer and autumn. The female cone enters the winter with no tissue differentiation of the cone body. This takes place very slowly but prominently in the course of the winter. The male inflorescence is well advanced in the autumn and winter progress is relatively slight.

The natural control of morphogenesis at the shoot apex including the fitful seeding habit of *P. resinosa* is considered in the light of the growth timetable. To explain its mechanism, a working hypothesis involving production, consumption, and concentration of auxins is advanced as a preliminary to future work.

I. Introduction

The apical meristem of the shoot in vascular plants derives scientific interest from its importance as the growth center at which originates the plant's axis and the primordia of the appendicular organs. These are all branches of one sort or another, morphologically regarded. The apex is thus the germ out of which the architecture of the shoot is elaborated, the point of origin of the characteristic structures of the vascular plant. As such it is necessarily the main generative center of the plant, a focus of both morphological and physiological interest.

Recently a good deal of attention had been paid to the gymnosperm apex (8) essentially because the gymnosperms are systematically intermediate between the angiosperms and the vascular cryptogams, the apices of which exhibit suggestive elements of difference. Studies such as that of Kemp (9) reflect a dual interest, combining a very close examination of the apical structure with studies of cellular behavior and with precise observations upon the timing of the morphogenetic cycles (0). Older work tended to concentrate upon the comparative anatomy of the apex (11) with less emphasis upon functional ideas. On the other hand, the subject of the present paper takes its interest mainly from physiological and ecological considerations, to some of which we have already recurred in the preceding papers of this series (4).

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The subject has also a certain practical importance arising out of the morphogenetic processes involved in the problematical seeding habit of *Pinus resinosa*. Long cycles of 8 years, more or less, of slight seed production or none are interrupted by short cycles of 1 or 2 years of high to moderate productivity. Such fitful activity undoubtedly deranges the natural regeneration of this important component of the forests of eastern North America. Though the species lends itself well to plantation forestry, the acquisition and maintenance of the necessary large seed stock is complicated in various ways by the uncertainties and irregularities of the natural seed production. From the forestry viewpoint we are thus confronted with the problem of discovering the factors in control of fructification in this species in the hope that enough might prove responsive to manipulation in such a way as to induce under the more or less controlled conditions of a seed orchard, if not in nature, a less problematical fruiting habit. As background to the design of any such program it is manifest that we require to know in some detail the time scale of the morphogenetic cycles at the stem apex. Though our interest in organogenesis is especially strongly engaged by those particular morphological branch equivalents which are the cones, nevertheless, the whole assemblage of parts represented by the primordia of a winter bud forms one integral system in which it is not possible to distinguish components which are negligible in their bearing on these researches from those which are not.

Ideally the subject demands an extensive enquiry involving thorough anatomical investigations not only of the various appendicular organs laid down successively as primordia in the bud but also of their subsequent development in extension growth. We have not been in a position to conduct such an investigation on the anatomical and morphological side without the sacrifice of too much of the physiological and ecological interest of these researches. Our objective has, accordingly, been limited mainly to the origination and development of the primordia in the bud and the timing of these events. Only the minimum histology has been attempted and we have touched but lightly upon the cellular detail of the differential mechanism and processes.

The following section of the paper gives a brief account of our procedures. This is followed by the main section in which the results are recorded and the paper then concludes with a brief discussion.

II. Materials and Methods

Buds were collected from trees growing in two places at which we established permanent observation stations. These were in the Ottawa Valley in the vicinity of Chalk River, Ontario, and at Craighurst in Simcoe County. The former is $1\frac{1}{2}$ degrees of latitude farther north than the latter and is more remote from the moderating influence of the Great Lakes upon climate. The growing season at Craighurst is, accordingly, from 1 to 2 weeks longer than at Chalk River. Collections were first made in the years 1946–47

when in both places, but especially in the vicinity of Chalk River, the seed trees of the species were in a phase of high productivity, and continued through 1950.

The collections were made at various times through the growing season as well as during late autumn and winter when growth is popularly supposed to have paused. The latter times are always included in the "resting periods" of the apices of other species (9, 14). We quickly found that the cycle of growth events in the bud and apex is, as far as we could judge, continuous throughout the year. Certainly differentiative growth within the primordium of the female strobilus, small in amount but of great morphogenetic importance, takes place in midwinter. A period of "rest", in which literally no growth of any kind occurs, may intervene somewhere, perhaps repeatedly, but if so, the period, or periods, must be short. Perhaps a "resting period" of conventional length may be thought of as occurring, but subject to occasional, intermittent bursts of activity, limited to certain centers within the bud and to certain times which external conditions dictate. In such circumstances it matters little whereabouts in the growth continuum our account of the morphogenetic cycle begins. We have therefore in Section III made the midsummer condition the starting point and have used a seasonal subdivision of the time scale as the most natural and convenient.

The collected materials were placed for fixation in formol acetic acid and subjected to vacuum infiltration at either the field laboratory at Angus or that at the Petawawa Forest Experiment Station. They were brought to Toronto in the fixing solution, there washed free of the fixing agent, and brought to 70% alcohol for preservation. We soon made the discovery that in large autumnal and winter buds the histological dehydration of the bud prior to embedding altered the tissue tensions of the bud scales so that after sectioning and rehydrating the bud scales forcibly opened out near their point of insertion on the axis while they curled up distally so as to overlie the section and impair observation and appearance (Fig. 18). We then routinely plucked off the bud scales while the materials were in 70% alcohol (Figs. 9, 14, 15). The material thus preserved was embedded by the tertiary butyl alcohol method in paraffin of melting point 56°–58° C., then cut in serial sections of about 10 μ, sometimes on a rotary, more often on a sliding microtome. Staining was with safranine and light green. This choice of procedure rested entirely upon our previous familiarity with it as applied to other subject material. Considering that we could not fully develop the histological side of the investigation in any event, we chose the time-saving rather than the time-consuming course and made no attempt to apply the procedures so carefully investigated by Ball (3) and used with such abundant success in beautiful preparations by other authors (9, 1).

The results were illustrated by a series of photographs and photomicrographs of which 23 of the latter are reproduced in the accompanying Plates I–IV inclusive. The captions accompanying each plate give full description of the corresponding figures and of the subject materials from which the photomicrographs were taken.

III. Results

III. 1. Summer Growth

Buds collected at Chalk River in July exhibit the apex in very active growth, though growth is, at this time, about to begin slowing down. Figure 1 is a photomicrograph at low magnification of a longitudinal section through an apical bud of July 21st. Figure 2 is a higher magnification of the dome of the same apex. The dome is multicellular in thickness, there being about six more or less uniform courses of cells and it is acute-angled or right-angled, not obtuse-angled. The beginnings of folds appear on each side of the dome. These will become bud scales. Further down the flanks fully developed bud scales may be seen. In the axillae of these scales, and restricted laterally to the mid-part of each scale, a small mass of meristematic tissue persists. This may be regarded as a direct derivative of the original meristem now isolated from it by the intervention of younger scales and occupying an increasingly lateral position on the bud axis as growth proceeds. The activity of the subsidiary meristem does not appear to diminish upon its separation from the advancing dome but continues unimpeded so that quite prominent mound-like secondary meristems are quickly distinguishable in positions close to the flanks of the main apical meristem (Fig. 1). Two such are seen at higher magnification in Fig. 3.

Folds presently appear upon the flanks of these lateral, secondary meristems and proceed to develop into secondary bud scales. This development converts the lateral secondary meristems into full-fledged lateral budlets. Further down from the apex in the bud that yielded Figs. 1 and 2, the budlet appearing in Fig. 4 occurred. Similarly lower in the bud from which Fig. 3 was derived the budlet shown in Fig. 5 was situated. Both buds were fixed on July 21st and the figures, taken in conjunction with the other sections of the series from which these came, show that to this date the meristematic part of the secondary budlet has not yet proceeded with the differentiation of the leaf fascicle primordium.

All the lateral budlets or their precursors shown (Figs. 1–5) are nevertheless destined to give rise to leaf-fascicle branches. The meristems of these budlets normally differentiate only budlet scales in the year of their formation. No leaf fascicles nor their primordia are formed until the following spring except on relatively infrequent occasions some of which have been observed to follow as the result of a complex induction by the artificial application of growth substances. On these occasions not only are the primordia of leaves formed in the more proximally placed budlets but these primordia proceed to undergo a limited extension growth. This occurs in the absence of appreciable extension growth in the main bud axis with the result that at the lower (proximal) region of the terminal buds, where the induced, premature growth of leaf fascicles chiefly occurs, leaves protrude from between the bud scales in the autumn. Terminal growth in the following spring from buds so affected is never quite normal and is frequently considerably distorted. We propose to restrict attention here to the usual instance, leaving the exceptional to be

considered in another paper in connection with the effects of the experimental application of growth substances.

Transverse sections through buds collected in the summer growth period, though slightly later than the collections from which Figs. 1 to 5 were taken, yielded Figs. 6 and 7. The former is a transverse section taken near the top of the dome at A–A in Fig. 2. The group of meristematic cells from the crest of the dome, with their enormous nuclei, appear quite clearly. Surrounding them are transverse sectional views of bud scales. Figure 7 is a transverse section at a lower level of the bud, corresponding to B–B in Fig. 1. Here we are below the dome and the section passes through the axis of the bud where secondary meristems corresponding to the earliest stage of budlet development (Figs. 1 and 3) are developing in spirals around the axis.

The relatively high proportion of tissue in growth to be seen in Figs. 1, 2, and 7 is characteristic of the high generative activity of the midsummer condition. The apices at other times when growth in the sense of primordium production is less active or when axial extension growth alone is in progress (as it is in early spring) present a quite different appearance and the dome a different form. One way of characterizing the form is to use the ratio of the height of the dome (from the apex to base) to the diameter of the dome at its base. For the stage here under consideration this ratio ranges from 0.43 to 0.50.

Figure 8 is from a transverse section of a bud fixed also on August 11th and is therefore representative of a late summer growth condition. This section is one of a series of which the one illustrated passes in a nearly median plane through a leaf-fascicle budlet. Taken in conjunction with the other sections of the series and with the similar results from the examination of the two series to which Figs. 4 and 5 belong, it is clear that the secondary meristem of the budlet remains quite undifferentiated except for budlet scales throughout the summer growth period. We shall find that they normally refrain from differentiating leaf fascicles or their primordia until the spring of the following year.

III. 2. Early Autumn Growth

Collections of buds made at Chalk River on August 27th gave the earliest indications of cone primordia, and sections from these are illustrated in Figs. 9, 10, and 11. In Fig. 9, which is a slightly off-median, longitudinal section through the bud axis and the apical meristem at low magnification, a considerable part of the lengthwise dimension of the bud is represented. The bud scales were plucked off before the buds were embedded and their stumps alone remain. The apical bud with its meristem is shown and on the flanks of the main bud axis the numerous leaf-fascicle budlets appear, forming a series of spirals around the axis. Hundreds of such budlets have been examined at this stage without finding any indication of leaf-fascicle differentiation.

On the shoulders of the main bud axis occur the two most recently produced budlets. These are the primordia of megasporangiate cones at the earliest stage at which we consider that we can distinguish them from leaf-fascicle budlets. There is no single, conclusive distinguishing characteristic at this

early stage but the distinction rests upon a conjunction of developmental stages or states in the apical meristem and its appendicular products. The uppermost products can be recognized as cone primordia when in the early autumn growth period a manifestly early stage of their own development, such as that in Fig. 9, is found associated in the bud with: (1) An evidently developing quiescence of the apical meristem. This is indicated readily by the flattening of the dome by comparison with that of the summer growth apex, the ratio of height to diameter at this stage being 0.23 (Fig. 10). (Cf. for Figs. 1 and 2 and similar domes, ratio 0.43 to 0.50.) (2) Contiguous leaf-fascicle budlets are much farther developed in the matter of budlet scale production than are the cone budlets. These points are not always demonstrable in any single section such as that of Fig. 9 and serial sections are required to make the matter clear.

Most of the leaf-fascicle budlets are produced like those illustrated in Figs. 1, 3, 4, and 5, when the apex is very active and exhibits a superficial cellular multiplicity such as that (ca. six-fold) shown in Figs. 1 and 2. In early autumn or late summer, depending upon latitude and season, as an accompaniment of rapidly declining growth activity in the apex, the six-fold (or higher) acute-angled dome gives way to a three-fold (or lower) obtuse-angled dome. Figure 10 illustrates the latter configuration of the dome in median section and we have already seen that its height–base ratio is only half that of the summer growth period dome of which Fig. 2 has already been examined as a corresponding example. Associated with the former configuration is the characteristic production of lateral subsidiary meristems in rapid succession so that they occur in relation to one another as we see them in Fig. 1 and in Fig. 3, with many leaf-fascicle branch primordia occurring contiguously at a very early stage of development with little or no production of budlet scales. It is necessary to go far down the flanks of the main bud axis at this stage before encountering even as considerable a budlet scale production as that shown in Fig. 3. The bud of Fig. 9 forms a marked contrast, the upper and latest-formed leaf-fascicle budlets being all more fully developed than the lower ones of midsummer buds such as those shown in Figs. 4 and 5. In these circumstances the identification as cone primordia of the circle of early-stage budlets surrounding a deactivating apex and surmounting late-stage leaf-fascicle budlets is not really hazardous.

Generally speaking, confirmation can be obtained from the structure and growth of the primordium itself. The reason for this is that the cone primordium continues growth throughout the autumn and even the winter, long after the apical meristem itself has ceased growth. Evidence of this in the form of advance preparation for growth in the cone primordium can be detected early. The young cone in the bud has a very considerable stalk (Figs. 14 and 15) and in the early stages of its development advance evidence of the forthcoming stalk can be inferred by the experienced observer from the evidences of activity in budlet scale production and of growth at the secondary apex itself, which is found to be quite acutely prolonged if examined at the

correct stage (Fig. 11). The axis and corpus of the embryonic cone also come to be well developed in the bud and advance evidence of this is also manifested early in the shape assumed by the distal extremity of the very young cone. The section from which Fig. 11 was taken illustrated these confirmatory points. There is actually a considerable provascular and prostalk development only a small part of which is included in the figure. The acute angle of the cone apex denotes a continuing activity of longitudinal growth which is in marked contrast with the form of the corresponding part of the contemporary and even earlier stages of the leaf-fascicle submeristem. The latter does not continue growth to the point of differentiating the leaf-fascicle primordia but remains in the meristematic stage over winter. This habit is associated with a very obtuse or even flattened apex which is recorded clearly in all the photomicrographs of these structures and appears accordingly in Figs. 4, 5, and 8.

These various evidences have enabled us to recognize the appearance of young cone primordia and thus to place the differentiation of the cone, to an approximation, in its proper place in the timetable of growth and morphogenesis. Accordingly, in the Ottawa Valley, in the vicinity of Chalk River, it would seem that the first steps of cone primordium formation are taken in mid-August and the young cones are identifiable as such in late August. This is the first overt step in the direction of seed production. It is manifestly important to know when the balance of factors promoting and opposing megasporangiate cone formation becomes critical and this now seems established with sufficient precision.

The question arises whether after the laying down of the cone primordium seed failure may occur as a result of cone abortion. We have never observed evidence of aborted cone primordia in any bud, though young cones sometimes fail at the time of emergence, for unknown reasons, or later for various reasons such as insect attack. We have examined many late August and September buds which were devoid of cone primordia. Such buds can easily be identified by hand lens or even with the naked eye after the bud scales are dissected away. Indeed, after a little experience the presence or absence of young cones in the September–October bud can be detected simply by palpation and when cones are present they can be counted in this manner 8 months before emergence. Numerous examinations from late August to October with the results here summarized have convinced us that seed crop failure and intermittent seed production are attributable in the first instance to failure in differentiating megasporangiate cone primordia in the late August bud of this species. Other causes of failure in seed production can result in substantial deficits from the full seed production capacity (12) of formed cones but cone failure itself remains the prime cause of recurrent, complete seed failure.

The cellular structure of the apex at this stage in its cyclic development is illustrated in Fig. 12. This section was taken from a bud collected at Craighurst on September 8th and represents the beginning of the transition from

the early to the late autumnal states and thus from diminishing generative activity in the production of primordia to no activity of this kind. The section shows clearly a superficial zone of initiation, a zone of subapical generative or mother cells, a pith rib meristem or pith mother cells, and a zone of flanking tissue. This structure is much more clearly manifest at this stage than at any other occurring before or after. The cellular multiplicity of the early autumn dome is considerably less than that of the dome at the midsummer or early primordium-forming stage (cf. Figs. 2 and 12). The volume of the dome is much less and the form much flatter (cf. h/d ratios of dome in Fig. 2 = 0.45, in Fig. 12 = 0.22). This architecture corresponds precisely to Sacher's description of the apices of *Pinus lambertiana* and *P. ponderosa* (14).

III. 3. Late Autumn Growth

The progress of events from September to November involves the virtual cessation of generative activity in the apical meristem, that is, cessation of primordium formation, but the continuance of growth in the cone budlets, certainly megasporangiate and possibly microsporangiate. Regarding first the apex itself, Fig. 13 is given as representative of the completed transition from early autumn to late. The section was taken from a bud collected at Craighurst on November 7th. Its structural features are clearly a simplification of those represented in Fig. 12, the antecedent stage. The superficial course of cells, which in Fig. 12 is closely similar to the underlying course, is in Fig. 13 in strong contrast to all other tissues of the apex in the large size of its cells and their nuclei and in their orientation. The superficial zone of initiation thus remains prominent but simplified, the group of subapical mother cells is much reduced in prominence, the cells being smaller and less different from surrounding tissues than they are in Fig. 12, and without evidence of activity. The rib meristem and the flanking tissues are also visible. The former gives evidence of residual activity but activity has completely ceased in the latter.

The form of the dome as well as the aspect of the cellular components is consistent with an almost completely inactive state. The dome with its lowered cellular multiplicity is also small and flat (the ratio h/d = 0.20), a condition that remains characteristic until the resumption of generative growth activity in the production of primordia late in the following June or in early July. At this time the axial extension growth of the primordia-bearing winter bud axis approaches its conclusion and the apical production of primordia for next year's extension growth commences (Fig. 23).

As the apex thus sinks into quiescence in autumn the cone primordia continue to grow and develop slowly. Figures 14 and 15 represent the condition in mid-October. The section illustrated in Fig. 14 is median longitudinal or nearly so with respect to the cones but not with respect to the apex. The dome is not intersected at all. The cone budlets present no difficulty in identification such as we encounter at their earliest stages. At the October

stage the megasporangiate cone is quite unmistakable, the cone stalk and the the corpus of the cone are clearly evident.

It is important to notice the rather large size of the October cone and the absolutely smooth surface of the cone body. This latter condition changes during the course of the winter. In order to stress the smooth condition of the October cone primordium a transverse section through the body of the cone is given in Fig. 16. This confirms the smooth condition of the surface at much higher magnification and demonstrates in addition that the degree of tissue differentiation in the interior of the cone is slight. It is thus manifest that the residual growth of the young cone in the middle and late autumn results in substantial enlargement and in the development of a stalk and body, but the tissues of the body present otherwise no evidence of further differentiation.

By contrast, the microsporangiate inflorescence achieves by early autumn (September 10th) a much higher degree of differentiation than that of the megasporangiate in October–November. Figure 17 represents a small portion of a longitudinal section through a bud of September 10th bearing both leaf-fascicle budlets and microsporangiate budlets. The figure shows the most proximally situated (lowest on the bud axis) leaf-fascicle budlet and the microsporangiate budlet immediately below it. The stalk, axis, and body of the male inflorescence are clearly evident and in addition the superficial tissues of the cone are sufficiently differentiated to embody the primordia of pollen sacs. The latter are substantially more advanced at the base of the microsporangiophore axis than they are at its apex. The superior advancement of morphogenesis in the male inflorescence to that in the female may, at least in part, be referable to the circumstance that in the "male winter bud" the differentiation of the inflorescence primordia at the apical meristem takes precedence in time over the differentiation of leaf-fascicle primordia whereas in the "female winter bud" the cone primordium is the last organ to be differentiated and follows after the long series of leaf-fascicle primordia. It is conceivable that this difference in order is related to the difference in the regularity with which the male and female inflorescences are formed. As we pointed out in Section I of this paper *P. resinosa* is characterized by an exceedingly fitful seeding habit which in the present section we attribute in the first instance to corresponding irregularity in megasporangiate cone formation. No such failures characterize the production of microsporangiate inflorescences though the production of microsporangia undoubtedly varies from year to year, nevertheless they have never been observed to fail completely or to be reduced to a vanishingly small production. The microsporangiate cone, laid down before the leaf-fascicle (or short-shoot) budlets, is not thus subject to the severe inhibitions that affect the megasporangiate cone. The latter is subject to complete failure for cycles of several years in succession and is laid down following the extensive series of leaf-fascicle budlets. The physiological significance of these circumstances will be reviewed in the fourth section of this paper.

We may record here that megasporangiate and microsporangiate cones occur together in the same bud very rarely indeed in *Pinus resinosa*. In the course of several years' field observations only six examples have been encountered.

III. 4. Winter Growth

The most interesting and unambiguous evidence of activity in morphogenetic growth during the winter period is to be found in the megasporangiate cone primordium. As we have already seen (Figs. 14, 15, and 16), the October–November condition of the cone in the bud is one of little tissue differentiation and a perfectly smooth body surface. Figures 18 and 19 show that by January this simple configuration has advanced. The cone has not changed greatly in size, though it has become slightly larger. More important than this small increase in size, however, is the fact that the surface of the cone body, in November quite smooth, has by January become covered with small protuberances. These are the early primordia of cone scales and their presence therefore marks an important development in morphogenetic growth. This observation establishes unmistakably the fact of midwinter growth activity. We have never observed cone scale primordia in the October–November bud. All fertile January buds manifest them. The amount of growth involved in this morphogenetic advance is so small and the kind of growth is so special that the question arises whether the differentiation of the cone superficies in *Pinus resinosa* is conditioned in any way significant for morphogenesis by the special and variable pattern of external factors under which it occurs. Certainly a considerable proportion of the cone scales of this species under our conditions turn out to be undeveloped and infertile (12). Whether this characteristic results in whole or in part from the conditions under which the cone scale primordia originate is a problem that future research must resolve.

III. 5. Spring Growth

The further elaboration of the cone scale primordia of January proceeds very gradually and by May 24th the condition, though considerably advanced, hardly suggests that a 4-month period has elapsed since the January condition obtained. Figure 20 is a transverse section which shows that the differentiation of the May cone scales is still essentially a relatively slight elaboration of a set of superficial protuberances. Nevertheless, below the surface the body of the embryonic cone is now developing in depth and a cortex and medulla are clearly discernible. From this point on, however, progress is rapid and by June 16th the young cones have emerged, with cone scales formed as in Fig. 21 and if not pollinated already, are at least ready for pollination. Figure 22 of the same date shows that internally the ovules are forming. The homogeneous cortex of May 24th has now become a series of well-formed cone scales and developing ovules. The cone at the stage of pollination constitutes a remarkable morphogenetic growth product for the relatively brief period of 3 weeks.

On June 16th the apex appears as shown in Fig. 23. The extension growth of the winter bud is by this time very well advanced but the apical meristem has evidently not yet commenced the production of the primordia for the forthcoming winter bud. Certain preliminaries to primordium formation have been accomplished, however, and these have to do with the size, shape, and cellular multiplicity of the dome and the resumption of bud scale formation. The dome is still small, but its shape has changed, as Fig. 23 shows, from the obtuse-angled form of Fig. 13 with a h/d ratio of 0.20 to an acute-angled form with a h/d ratio of 0.43. Thus the June apex combines the form and certain characters of the fully active July apex with the practically inactive apex of November. This brings us around to the July condition with which this account began.

IV. Discussion

IV. 1. The Timetable of the Morphogenetic Cycle

Period of Primordium Formation

The facts recorded in the preceding section demonstrate that the laying down of the organ primordia with which the terminal winter bud is furnished occupies the latter part of the "growing season" consisting roughly of the 2 months of July and August with perhaps brief periods at the end of June and the beginning of September for the preparatory and terminal stages of this generative activity. The primordia thus formed in the megasporangiate cone-bearing bud are, in order of formation, those of leaf-fascicle branches (short shoots), lateral branches (long shoots), and cones, all in the form of buds. In microsporangiate buds the primordia laid down are those of microsporangial cones, leaf-fascicle branches (short shoots), and lateral branches (long shoots) in that order. The formation of microsporangiate and megasporangiate cones in the same bud as we have seen occurs exceedingly rarely and almost invariably the male and female morphogenetic cycles originate in separate buds and hence are passed through upon separate shoots. This separation in time and space is accentuated by the occurrence of reverse gradients in the frequency of male and female shoots along the axis of the tree. Male and female shoots are found in all zones of the tree but male shoots are most frequent in the lower zone and least in the upper zone while the reverse is true of female shoots.

We shall presently show in another paper that in young trees capable of seed production, the onset of axial extension growth in May or late April begins first in the upper and last in the lower zone of the tree while its conclusion in June–July takes place in the reverse order. The beginning of primordium formation occurs just as axial extension growth concludes. Hence primordium formation begins first in the lower zone where male shoots are the predominant type of fertile shoot. In the male bud the primordia to be laid down first are the male primordia. Thus the laying down of the microsporangiate cones in the lower zone of the tree has some advantage in time over the formation of megasporangiate cones, especially those in the upper

zone. These cones not only occur in the buds which begin primordium formation a little late but their cone primordia themselves are the last to be formed at the main bud apices.

Growth in the "Resting Period"

The phase of primordium formation ends in September and the apex becomes quiescent and desists from generative activity from October through January to and including May. The secondary buds or budlets representing the primordia of leaf-fascicle branches (short shoots) and those representing lateral branches (long shoots) normally also remain quiescent and without evidence of generative activity in the production of primordia during most of this period. But the megasporangiate cone primordium undergoes progressive development though it is uncertain whether this occurs as a very slow continuous or almost continuous process or in bursts of more considerable activity well separated in time. However this may be, the result of this localized activity is that the externally smooth and internally homogeneous primordium of October (Figs. 14 and 15) is by January superficially tuberculated but internally still undifferentiated (Figs. 18 and 19). By late May it is differentiated in depth to form a cortex which will develop into cone scales and ovules and a medulla which will become the cone axis (Fig. 20). The period October to May is designated by most authors considering the apex of other species of gymnosperms as a "resting period" (9) but we must make an exception of the megasporangiate cone primordium in *Pinus resinosa*. The microsporangiate cone, which by early September in this species reaches an advanced state of differentiation (Fig. 17) corresponding to the May–June condition of the megasporangiate cone, remains relatively if not absolutely inactive during the winter months.

Period of Axial Extension Growth

Following upon the period of winter growth, in which interest attaches chiefly to the progress of the megasporangiate cone, comes the period of axial extension growth. This occupies the period May, June, and early July, thus overlapping the "resting period" of the apex. This is possible because the chief activity of the axial extension growth is the elongation or extension of the axis of the winter bud and of the primordial branch equivalents with which it is furnished, all of which bears now an indirect rather than direct structural relation to the apex.

Early in this period the axis of the winter bud begins to grow longer, the bud swells, the bud scales separate from one another and begin to fall away as the lengthening process continues. This process of extension growth, when measured by the length of the bud axis and its extension at every stage, traces a sigmoidal pattern along the time scale. We propose to examine this aspect of the tree's growth formally elsewhere. In this paper it must be treated incidentally and employed simply as an index of the progress of terminal growth in time.

In mid-June, when the axial extension growth of the shoot is about half accomplished and is at its maximum activity, the apex takes on the acute-angled form characteristic of generative activity as shown in Fig. 23. But the active formation of primordia by this apex is still about three weeks in the future, during which time the dome increases its cellular multiplicity and the apical outline assumes the configuration of Fig. 1. Considering how great is the activity of extension growth during this period the relative inactivity in generative growth of the apex that caps the growing shoot seems remarkable and suggestive of some powerful inhibition at work. Of the two species of pine examined by Sacher (14), in *Pinus lambertiana* generative activity begins in the apex somewhat before extension growth has ceased whereas in *P. ponderosa*, as in *P. resinosa*, extension growth ceases before generative growth in the apical meristem resumes. Amongst the gymnosperms at large the latter habit seems more frequent than the former (9, 15), but more data are needed to make the position quite clear, especially amongst the pines. This is a point that bears strongly upon the control mechanism of morphogenesis at the shoot apex.

During the early and middle parts of the period of axial extension growth there is a great acceleration of growth in the leaf-fascicle branch budlets, the lateral branch budlets, and the male and female cone budlets. Cones, both male and female, develop rapidly, undergo extension growth, the cones emerge, and pollination occurs well before axial extension growth is concluded. Important morphogenetic events are therefore occurring at a time when the generative activity of the apex (primordium development) is low or absent. It is true of the morphogenesis of the female cone that practically the whole of it up to the time of pollination occurs during two intervals both of which are characterized by low apical activity. These intervals are the period of winter inactivity of the apex and the period of axial extension growth most of which is also marked by apical inactivity. The passage of the apex from the condition of Fig. 23 to that of Fig. 1 occurs in July. This marks the transition from the period of axial extension growth to that of primordium formation with which we began.

IV. 2. The Architecture of the Apex and Its Modification with Progress of the Morphogenetic Cycle

The work of various authors on this subject in members of the Pinaceae has been summed up by Johnson (8) by saying that two types of growth sequence are to be recognized; these we may refer to as the complex and the simple. In the former the architecture of the apex is characterized by a manifest zonation in which usually four centers of generative growth are recognizable. These vary in appearance as the morphogenetic cycle progresses; the distinctions between the several centers are in some stages less manifest than in others and the apex concurrently undergoes marked changes in size and shape. This kind of growth sequence is ginkgoid in type and seems to be frequent in the genus *Pinus* (14) as well as in other genera of the Pinaceae (8).

The second type is characterized by much greater uniformity. The zonation of the apex is always less manifest than it is at the times of maximum development in the complex type and changes in apical volume and shape are minimized. It is clear from the account given here in Sections 3 and 4 of the structure of the main shoot apex in *Pinus resinosa* and the variations the apex undergoes as the morphogenetic cycle is traversed that this species is a representative of those characterized by the complex type of morphogenetic growth sequence. The genus *Pinus* contains representatives of both types (10, 14). Other genera such as *Torreya* (9), *Pseudotsuga* (2, 14), *Abies* (5, 10), and *Picea* (10) belong to the complex type; *Pinus montana* (10), to the simple type.

The apex of *Pinus resinosa* is undoubtedly in its most active state during the period of primordium production and a comparison of Figs. 1, 2, 10, 12, 13, and 23 not only indicates this fact but also demonstrates visually the range of modification that the apex undergoes as the morphogenetic cycle advances. This purely visual record can be usefully supplemented by reference to the numerical results of measurements made on sections of the apices corresponding to those illustrated in the photomicrographs. These results are assembled in Table I in which are given the growth period, the corresponding growth activity of the apex, the dimensions of the apices as diameter of the dome base and as height of the dome apex from the base, all in millimeters, the volume of the dome in cubic millimeters, and its shape or elevation as represented by the value of the ratio: height/diameter of base.

TABLE I

MEASUREMENTS* OF CERTAIN DIMENSIONS OF THE MAIN SHOOT APEX OF YOUNG SEED TREES, WITH DERIVED NUMERICAL INDICES OF THE VOLUME AND SHAPE OF THE DOME IN RELATION TO THE CORRESPONDING GROWTH PERIOD AND ITS APICAL ACTIVITY

Fig. No.	Growth period	Apical growth activity	Linear measures of dome, mm.		Volume of dome, mm.^{3}	Shape of dome; h/d
			diam. (d)	height (h)		
Fig. 2	Summer (July)	Primordium production active	0.69	0.310	0.0353	0.43 0.50
Fig. 10	Early autumn (August)	Primordium production concluding	0.46	0.114	0.0189	0.23
Fig. 12	Transition (Sept.)	Primordium production concluded and	0.41	0.0930	0.00389	0.22
Fig. 13	Late autumn (Nov.)	apical meristem recovering	0.57	0.114	0.0103	0.20
Fig. 23	Spring (June)	Apex reactivating	0.54	0.230	0.0176	0.43

*All measurements were made upon stained preparations and no attempt has been made to correct for shrinkage or distortion that may have been induced by the histological procedures.

It is clear from Table I that very considerable changes in the size of the apex take place, the variations of height being greater than those of diameter at the base. The range of the variations these induce in the volume (or mass) of the apex is approximately 10-fold. It is clear that the mass of generative cells and their immediate associates in the apex is maximal at the beginning of the period of primordium production in middle and late July and from that point falls away to a minimum as this important function proceeds, reaching the minimum in early September by which time the generation of primordia is quite finished. Thereafter there is some suggestion of a recovery from the very low minimum of September to a low (but not minimal) winter condition corresponding to continued zero generative activity in the apex. Kemp (9) suggests that such sequences develop as the result, ultimately, of the exhaustion of "growth factor" supply. This seems to be a valuable idea and comports fully with the hypothesis that will be outlined in the concluding part of this discussion to account for control of morphogenesis at the apex. It is interesting that minimum size of the apex is reached simultaneously not only with the conclusion of primordium generation and with the onset of apical inactivity but also with its least elevated form (Table I, column 7) and with its clearest manifestation of the four types of generative cells that constitute the essential working part of this apex (Fig. 12).

In the spring the most prominent early growth activity is the extension growth of the winter bud axis. This is accompanied by the development and outgrowth of the primordia of the lateral short shoots (leaf-fascicle branch budlets), the lateral long shoots, and of the cone primordia. This activity occupies the months of May and June and some part of July. It is a tremendous growth but it involves the generation of no new primordial structures, merely the exploitation in growth and development of components some of which were first generated as primordia as much as 9 months previously. Throughout this exceedingly active period the apex remains entirely inactive in the generation of primordia. It does, however, augment in size and also increases its elevation to a value indistinguishable from that (0.43 to 0.50) which characterizes the apex at the beginning of its generative activity. In late June the size increase has brought the apex to a volume of about half the maximum size. The latter is then reached about a month later in the early stages of primordium generation.

IV. 3. Control of the Morphogenetic Cycle

The mechanism by which the morphogenetic cycle is controlled so that, for instance, when one kind of growth (such as primordium production) is proceeding another kind (such as axial extension growth) is inhibited is in this instance quite unknown. But in other and different instances it has been found that experimental evidence can be adduced for the control of a complex terminal growth (6, 7) by the production, consumption, and concentration of auxins. Generally speaking this type of hypothesis has proved to be valuable in our present state of knowledge. For this reason in part and partly also because it is so clearly accessible to experimental verification a

hormonal hypothesis of the control of morphogenesis at the shoot apex is outlined here as a preliminary to further investigation of the present species.

In the present instance we have to do with the mutual and reciprocal effects upon each others' activity of five different kinds of generative growth centers and one area of extension growth in the apical winter bud. These centers are the main apical meristem itself surrounded and flanked proximally by four additional kinds, all taking the form of small, secondary, lateral budlets distributed in a characteristic pattern along the main bud axis. The area of extension growth is the bud axis itself. The four generative centers additional to the main bud apex are: (1–2) two kinds of lateral shoot budlets, the short shoots (leaf-fascicle branches) and long shoots (lateral branch budlets), the prime characteristic of which is that they remain for months as undifferentiated buds consisting of meristem and bud scales only; (3) microsporangiate cone budlets, in which the budlet meristem produces the primordia and these develop well-advanced microsporangiate cones within a short time after the budlet is laid down; (4) megasporangiate cone budlets, in which the budlet meristem proceeds with the production of the cone primordium immediately but slowly, along a prolonged time scale. This cone primordium does not pause in its development but slowly differentiates superficially on the cone body the primordia of cone scales. These remain advancing slowly in this superficial and preliminary stage during the winter with slow differentiation of the inner tissues of the cone body to form a cortex and medulla by spring. The formation at the apex of numerous primordia in the manner described strongly suggests that in the process the auxin supply available to the apex is seriously depleted in primordium formation (9), in this species probably to the point of complete exhaustion. Primordium formation would thus, on hypothesis, occur during a time of progressively diminishing auxin supply and when extension growth in the main bud axis, which must be regarded as the chief local source of auxin production at about this time, has ceased entirely.

The dormant budlets, whether of short shoots or long shoots, would be expected to produce and to contain little or no diffusible auxin (6) as long as they remained in their original early condition. In this state they can be regarded as net sinks rather than net sources of growth substance. In the capacity of sinks they would be capable, through competition for growth substance, of exerting an inhibitory effect upon the growth of any adjacent center which is actuated by as high a concentration of auxin as they themselves require. The very large number of short-shoot budlets laid down in a large apical bud could act in this way during the autumn and winter as a powerful competitive inhibitor and control of terminal growth in all its aspects. There is nothing to guide us in forming an opinion as to the auxin potentialities of the fertile budlets. But it is manifest that the auxin potentiality of all five types of generative growth centers in the winter bud can be determined experimentally and the conclusions given in this subsection can be confirmed or modified by experiment at every stage of the morphogenetic cycle.

The series of short-shoot budlets would be unable to inhibit initially the microsporangiate budlets because the latter are formed before the short-shoot budlets appear. If the microsporangiate budlets were in a position to produce or acquire auxin—and it is evident that they would be formed while auxin production from the extension growth of the main bud axis was still presumably appreciable—it would be possible for them to continue growth and development as in fact they are seen to do. On the other hand, the short-shoot budlets are produced before the megasporangiate cone budlets appear and the former are therefore capable of exerting an initial inhibitory effect upon the latter. If this inhibition were sufficiently severe it could be held responsible for the failure of megasporangiate budlets to be formed at all. As we have seen, cone failure is very much more frequent than cone production in this species. If the inhibition were less severe megasporangiate cone production could occur but with varying degrees of cone scale fertility and the very slow development of the primordium that we have witnessed could continue under control of the moderate inhibition in the manner in which we have seen that it actually continues during the winter.

In spring a considerable production of auxin by the main axis of the bud as it passes into extension growth may be postulated with some confidence. This would relieve the competitive inhibitions by supplying auxin locally and lead to active growth on the part of the four types of growth centers present in the winter bud. Each of the four as it entered upon the phase of its own extension growth would pass into a state of surplus auxin production, thus being transformed from a sink to a source. So each center would contribute to the activation of terminal growth as a whole which is, as we have seen, phenomenally active at this time (4). When these integrated activities had resulted in producing the terminal long shoot, the lateral long shoots, the numerous leaf-fascicle short shoots, the development of any cones that might have been laid down, and the occurrence of pollination, the production of auxin would be expected to decline. The passing of the main apical meristem into the phase following that of primordium generation and its continuance in that phase would result from the progressive decrease of diffusible auxin production.

In a survey not only of this cycle but also of the morphogenetic cycles of other species, the strong impression is sustained that primordium production certainly accompanies and may depend upon local low auxin supply and (what may be effectively the same thing) cessation (or at least some degree of slowing down) of extension growth. Whether or not the causal relations are here properly conceived future work can decide. At least the facts as known for this species concord with the conclusions. As the leaf-fascicle (short-shoot) budlets begin to accumulate on the flanks of the newly forming bud and to exert a progressive inhibition, the production of auxin by the almost fully extended terminal shoot continues to decline. Thus a strong, local state of mutual competitive inhibition would again rapidly build up, finally threatening or achieving complete inhibition of the formation of

megasporangiate cones. If cones are formed the genesis of sporophylls may be impaired by critical auxin deficiency and finally ovule formation and development may be inhibited with serious loss of fertility on the part of formed cones. Thus the exhaustion of diffusible auxin production by the generative activity of the apex would bring in its train the various consequences that have here been found to characterize morphogenesis at the shoot apex of this species.

Acknowledgments

Once more the authors acknowledge their indebtedness to the authorities, originally of the Ontario Department of Lands and Forests and of the Research Council of Ontario for grants that initiated these researches. The Departmental Committee for Extra Mural Research Projects, Canada Department of Agriculture, is currently supporting this work. The authors are grateful to the leaders of the Division of Forest Biology for their sponsorship and to the Chairman and Members of the Committee for their comprehending interest in every aspect of this work. Professor Bruce W. Kelley very kindly made collections for us on occasions when it was impossible for us to be in the field. Doctors D. H. Hamly and K. H. Rothfels lent us photomicrographic equipment and were generous with their time in giving advice and assistance.

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Explanation of Figures

Plate I

Fig. 1. Median longitudinal section of apical meristem. July 21, 1949, Chalk River. (×40)

Fig. 2. Median longitudinal section of apical meristem. July 21, 1949, Chalk River. (×110)

Fig. 3. Longitudinal section below the flank of the meristem showing bud scales and axillary submeristems. July 21, 1949, Chalk River. (×105)

Fig. 4. Longitudinal section of same bud as Figs. 1 and 2, further down the axis in proximal direction showing secondary leaf-fascicle or short-shoot budlet with its own meristem and primordia of budlet scales. July 21, 1949, Chalk River. (×115)

Fig. 5. Longitudinal section of same bud as that of Fig. 3, showing median section of axillary, secondary, leaf-fascicle, or short-shoot budlet with its own meristem and primordial budlet scales. July 21, 1949, Chalk River. (×115)

Fig. 6. Transverse section through bud at A–A in Fig. 2, showing section near tip of the dome surrounded by sectional views of bud scales. July 21, 1949, Chalk River. (×110)

Plate II

Fig. 7. Transverse section through bud at B–B in Fig. 1, showing section of axis below the dome with secondary meristems corresponding to early stages of leaf-fascicle or short-shoot budlets developing in spirals around the axis. July 21, 1949, Chalk River. (×40)

Fig. 8. Median transverse section through a leaf-fascicle or short-shoot budlet in late midsummer condition demonstrating a completely undifferentiated state of the budlet meristem at this date. August 11, 1949, Chalk River. (×115)

Fig. 9. Longitudinal section of bud almost median through the apical meristem with numerous leaf-fascicle budlets on the flanks and two early stage cone primordia on the shoulders. The cone primordia exhibit stalk and cone body, the leaf-fascicle budlets are quite undifferentiated. Bud scales plucked off except immediately over the apex. August 27, 1946, Chalk River. (×15)

Fig. 10. Median longitudinal section showing apical meristem in declining activity and exhibiting the characters of this state, in particular the obtuse-angled dome of the resting meristem in process of development (cf. Fig. 2). August 27, 1946, Chalk River. (×105)

Fig. 11. Median longitudinal section of very young cone with advance evidence of forthcoming body and cone-stalk formation. August 27, 1946, Chalk River. (×105)

Fig. 12. Median longitudinal section of apex beginning the transition between early and late autumnal state and from diminishing meristematic activity to none; showing also the centers of generative growth: S—superficial initiating cells; SA—subapical generative or mother cells; R—pith mother cells or pith meristem; F—flanking cells. September 8, 1950, Craighurst. (×105)

Plate III

Fig. 13. Longitudinal median section of apical meristem which has ceased activity. The dome is obtuse-angled and exhibits a single superficial course of cells differing in size and orientation from those below. Other centers of generative activity visible but less clearly than in Fig. 12. November 7, 1950, Craighurst. (×110)

Fig. 14. Longitudinal section of bud median through two cones. The cone budlets are intact, the cone bodies and stalks are well developed. The surface of the cones is smooth and the interior tissues homogeneous and undifferentiated. October 17, 1948, Chalk River. (×12.5)

Fig. 15. The right-hand cone of Fig. 14 at higher magnification. (×25)

Fig. 16. Transverse section through cone body and surrounding budlet scales. Interior of body is undifferentiated. October 17, 1948, Chalk River. (×110)

Fig. 17. Longitudinal section bud contrasting the state of advancement observed in leaf-fascicle budlet with the meristem characteristically not producing any leaf primordia and the male cone budlet with characteristically advanced differentiation reaching delimitation of pollen sacs. September 10, 1950, Chalk River. (×50)

PLATE IV

FIG. 18. Longitudinal section of bud with young cone budlets in median and near-median section. The surface of the cone body is now covered with protuberances which are the primordia of cone scales, tissues otherwise undifferentiated (cf. Fig. 14). January 1947, Chalk River. (×10)

FIG. 19. Right-hand cone of Fig. 18 at higher magnification. January 1947, Chalk River. (×25)

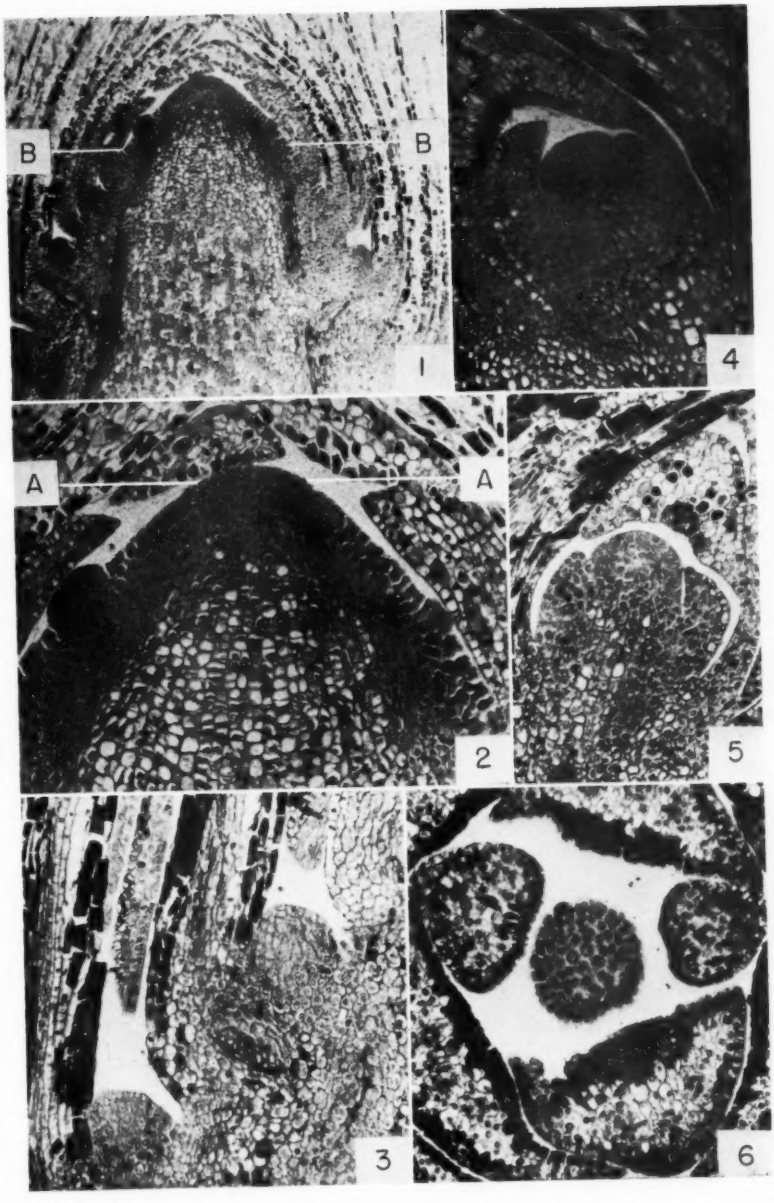
FIG. 20. Transverse section through cone body in budlet, with budlet scales surrounding. The interior of the cone body is now differentiated into medulla and cortex, the latter showing the protuberances (cone-scale primordia) considerably developed from the January condition and evidently becoming organized into cone scales. May 24, 1947, Chalk River. (×40)

FIG. 21. Longitudinal, tangential section of young emergent cone showing the highly organized condition of the cone scales. June 16, 1947, Chalk River. (×35)

FIG. 22. Median longitudinal section of emergent cone showing the ovules in process of organizing. June 16, 1947, Chalk River. (×25)

FIG. 23. Median longitudinal section of apical meristem showing the multiple, acute-angled dome. June 16, 1947, Chalk River. (×110)

PLATE I



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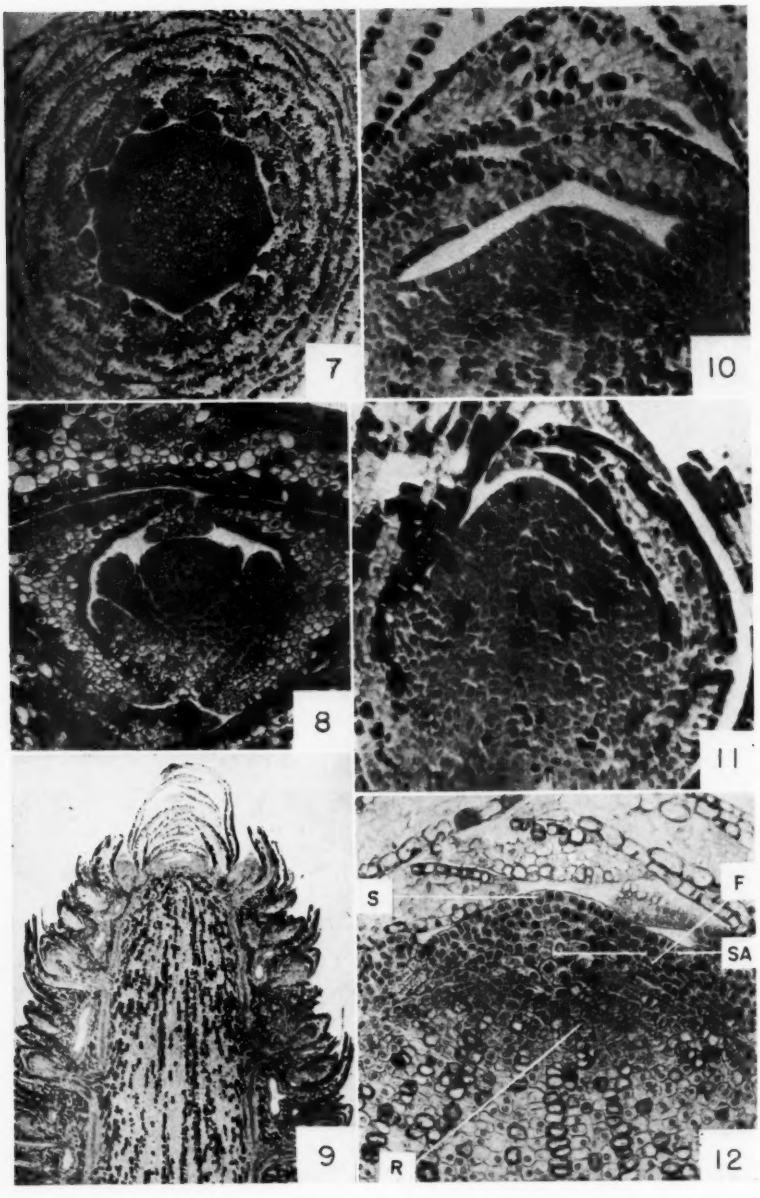

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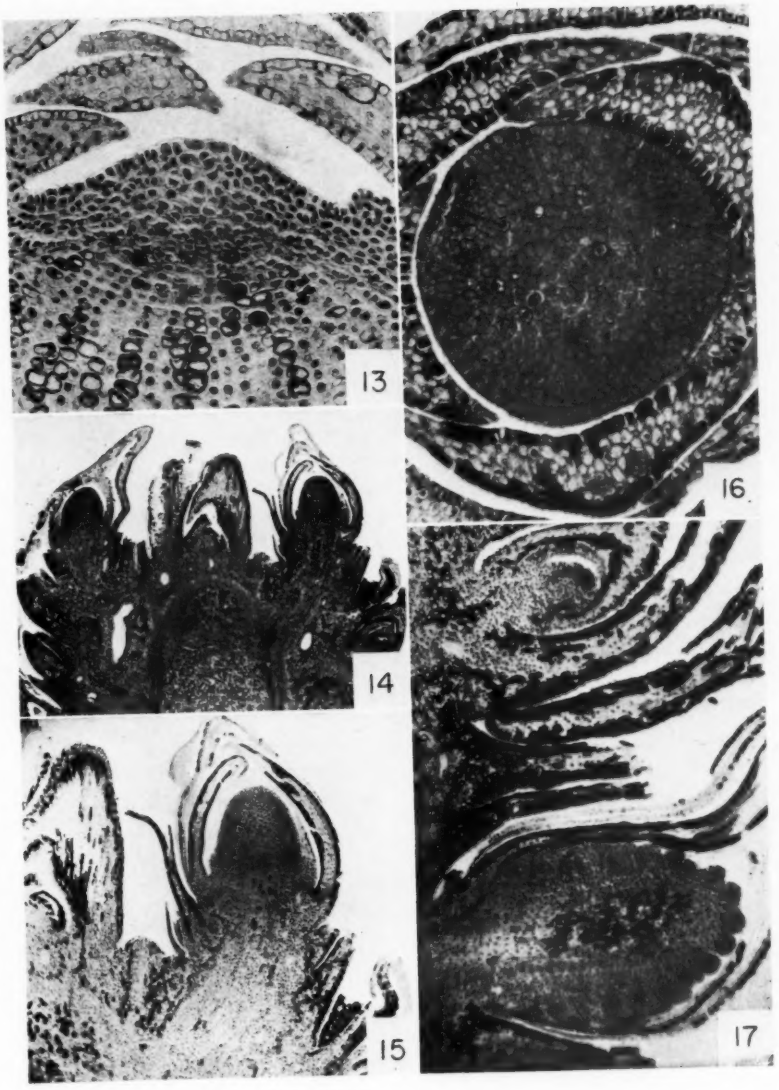
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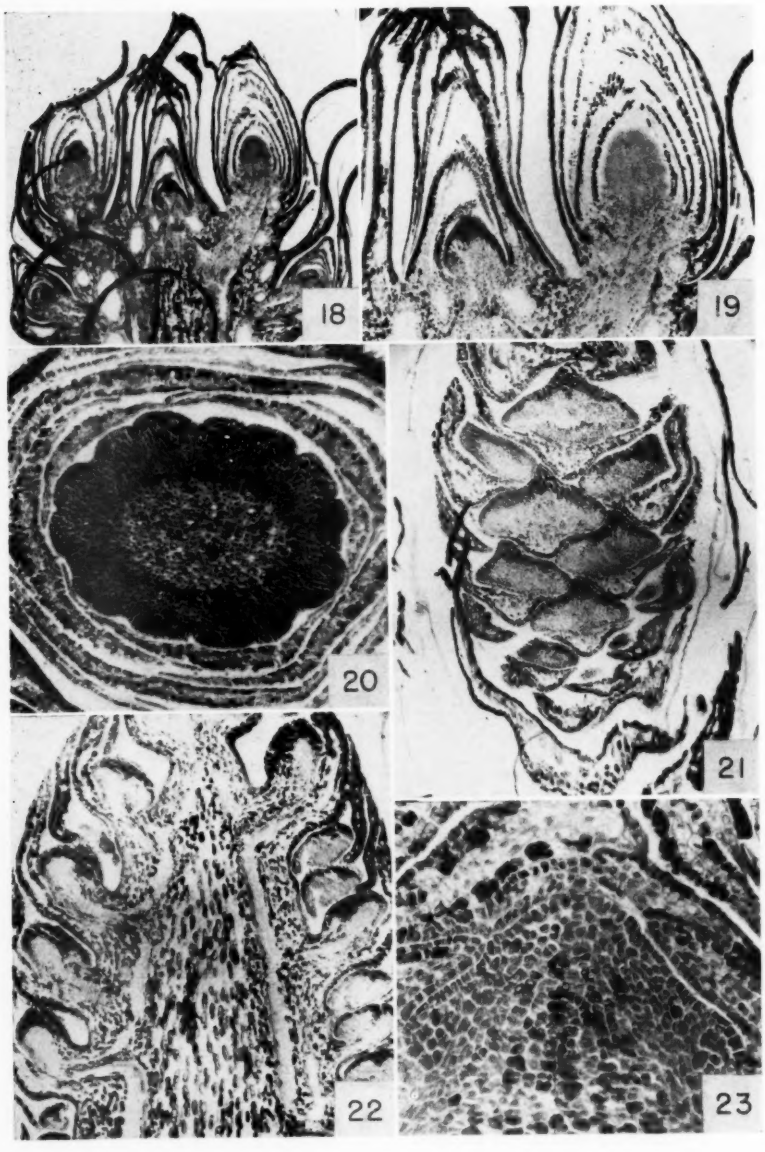
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PHOSPHATE METABOLISM IN LEAVES

I. IDENTIFICATION OF SOME PHOSPHORYLATED COMPOUNDS IN WHEAT LEAVES[1]

V. C. RUNECKLES[2]

Abstract

The phosphorylated compounds present in wheat leaves have been investigated following their extraction from leaf tissue supplied with radioactive orthophosphate (P^{32}). In addition to several glycolytic and photosynthetic intermediates, the compounds identified include the phospholipid constituents, phosphorylcholine, phosphorylethanolamine, and their L-α-glyceryl esters.

Alkyl phosphates have been shown to appear as artifacts in extracts obtained by using alcohols as killing agents. A mechanism for their synthesis is discussed, together with their importance in the interpretation of data on the dynamics of phosphate metabolism in vivo.

Introduction

The biochemical investigations of the past decade have emphasized the important role of phosphorus in intermediary metabolism. Thus the interconversions of sugars, their breakdown by glycolysis or the monophosphate shunt, and their production by photosynthesis all involve phosphorylated intermediates, while many of the endergonic reactions occurring in plants and animals require energy from the so-called "energy-rich" bonds of compounds such as adenosine triphosphate.

The bulk of our knowledge of phosphate metabolism has been obtained from such studies, wherein emphasis has been placed upon changes of the non-phosphatic portions of the compounds involved, rather than on pathways of phosphate metabolism as such. In such studies, the phosphate groups of the compounds involved have been regarded as essential but incidental components of their molecular structure.

However, Goodman *et al.* (5) have investigated the phosphorus metabolism of *Scenedesmus* using phosphorus-32 supplied as orthophosphate. Recently, Bradley (3) has extended these studies in investigating the transient phosphates produced by the photosynthetic process. Grube (6) and Simonis and Schwinck (16) have investigated the phosphorus metabolism of *Ankistrodesmus* and *Elodea*, Tolbert and Wiebe (18), the phosphate constituents of xylem sap, and Benson and his co-workers have shown the occurrence of phosphorylcholine and diglyceryl phosphate in plants (11, 12).

The work to be described was undertaken to obtain information on the compounds present in wheat leaves which become labelled with P^{32} when supplied with radiophosphate.

[1]Manuscript received June 17, 1958.
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Methods

Thatcher wheat plants were grown on a 3:1 plotting soil – vermiculite mixture. Sixteen days after germination the primary leaves were completely expanded (10) and were selected for experimental use. P^{32} was supplied in distilled water, either by vacuum infiltration, or by allowing the leaves to take up solution through their cut ends.

At the completion of an experiment, the leaves were first washed to remove adhering phosphate, and then killed and extracted with boiling 80% ethanol or methanol for 10 minutes. The alcoholic extract was decanted and the residue extracted with boiling water for a further 10 minutes. The extracts were combined and evaporated to dryness at 30° C. *in vacuo*. The dry residue was washed with two 5-ml. portions of dry toluene to remove fatty materials, and then dissolved in a known volume of water and applied to paper. Fractionation of the extracts was effected by the ionophoretic technique of Runeckles and Krotkov (14). Subsequent autoradiography of the chromatograms employed either DuPont Photoroentgen or Kodak "No Screen" X-ray film.

For co-chromatographic studies, the following systems were used for development:

1. *t*-butanol:picric acid:water (80 ml.:2g.:20 ml.) (7).
2. 2-butanone:picric acid:water (90 ml.:2 g.:10 ml.).
3. Pyridine:ammonium hydroxide:*iso*-propanol (4:2:1) (4).

Radioactivity determinations were made with an end-window G-M tube directly on samples on paper, or on aluminum planchets after elution and plating. Orthophosphate was determined by the method of Allen (1).

Results and Discussion

Identification of Compounds

The multiplicity of naturally occurring phosphorylated compounds in plant tissues has been shown by several reports (e.g. 2, 8). Figure 1 illustrates the large number of compounds labelled with P^{32} which can be extracted from green wheat leaves. A tentative identification of many of these compounds is immediately possible by virtue of their relative positions on the autoradiogram. Thus the identities of ribose-5-phosphate (R5P), glucose-6-phosphate (G6P), fructose-6-phosphate (F6P), 3-phosphoglyceric acid (3PGA), phosphopyruvic acid (PPA), adenosine-5′-phosphate (AMP), adenosine-5′-diphosphate (ADP), adenosine-5′-triphosphate (ATP), uridinediphosphoglucose (UDPG), and free orthophosphate (P_i) were readily confirmed by co-chromatography with authentic samples. At least 18 other P^{32}-labelled compounds were present in most extracts. The locations of these are also shown in Fig. 1.

Most of the phosphates were present only in submicrogram amounts, rendering the levels of their non-phosphate moieties too low for chemical characterization. Consequently identification of some of the unknowns has been based on their co-chromatographic properties, and their rates of hydrolysis

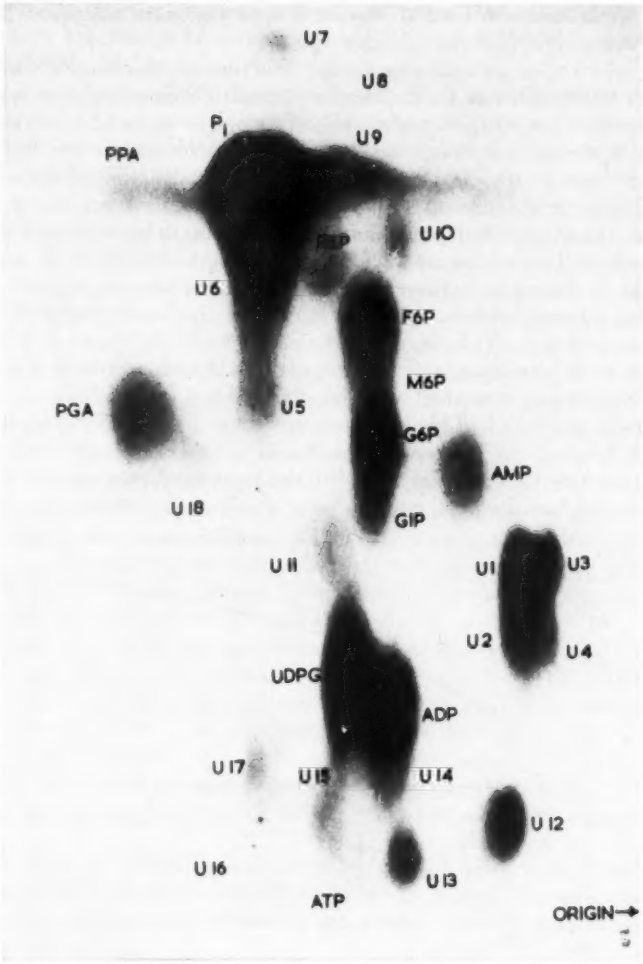


FIG. 1. Autoradiogram of wheat leaf extract. Leaf tissue killed in boiling ethanol after 60 minutes in light after infiltration with radiophosphate.

in acid or alkali. Attempts to obtain corresponding compounds labelled with carbon-14 by allowing wheat leaves to undergo prolonged photosynthesis in the presence of $C^{14}O_2$ were unsuccessful because of the difficulty of separating the small amounts formed from traces of the more abundant labelled metabolites.

Some information as to the molecular structure of the compounds present has been obtained from their ionophoretic and chromatographic behavior. In the pyridine – acetic acid buffer used for ionophoresis (pH 6) (14), mobility varies with the relative charge density of the molecule, an approximate function of the number of ionizable groups and the molecular size. For chromatography in the methyl cellosolve – pyridine – acetic acid – water developer (14), R_F values bear an approximately inverse relationship to both number of ionizable groups and molecular size. Thus a compound such as phytic acid migrates rapidly during ionophoresis, and slowly during chromatography, while 3PGA has a comparable ionophoretic mobility but a much higher R_F value. Similarly, ATP has a higher mobility than ADP but a lower R_F value. These properties have been of assistance in the identification of some of the unknowns.

The first compound studied was that depicted in Fig. 1 as U 1. The work of Maizel *et al.* (11) had shown the occurrence of appreciable amounts of phosphorylcholine (PC) in barley seedlings. The ionophoretic behavior of U 1 suggested that it was PC, for at pH 6 this compound possesses a zwitterion structure and hence would migrate only slowly. Co-chromatography and

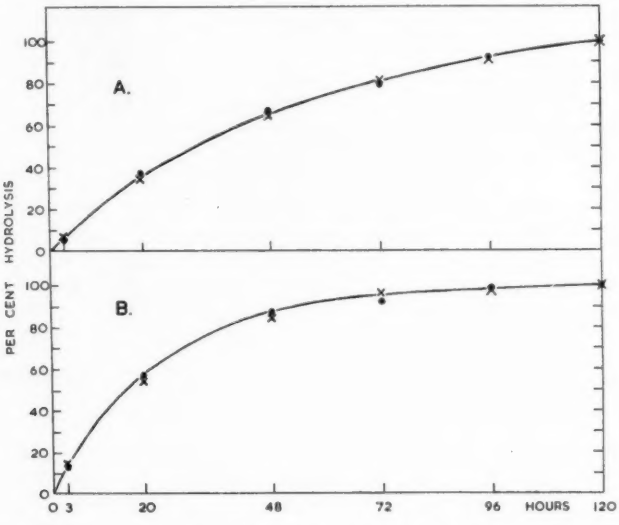


FIG. 2. Hydrolysis curves for (A) phosphorylcholine and U 1, and (B) phosphorylethanolamine and U 2, in *N* HCl at 100° C. Values expressed as percentages of 120-hour value. Solid circles: chemical determination of orthophosphate release; crosses: radioactivity determination of P^{32}-orthophosphate release, after ionophoretic separation of reaction products.

ionophoresis with authentic phosphorylcholine yielded only a single spot in all systems used. Its identity was confirmed by acid hydrolysis of the labelled unknown in the presence of carrier PC. The release of radioactive orthophosphate, determined after ionophoretic separation from residual PC, agreed closely with the release of orthophosphate determined by chemical means, as shown in Fig. 2.

In close association with U 1 are the three spots U 2, U 3, and U 4 in Fig. 1. Phosphorylethanolamine (PE) was found to co-chromatograph exactly with U 2 and to possess an identical acid hydrolysis rate (Fig. 2).

During ionophoresis U 3 and U 4 migrate from the origin at the same rate as non-ionic compounds such as glucose (as a result of electroendosmosis and capillary flow of buffer through the paper) suggesting their electrical neutrality at pH 6. The glyceryl esters of PC and PE possess no net charge at this pH because of zwitterion formation as shown in Fig. 3. Samples of authentic L-α-glycerylphosphorylcholine (GPC) and L-α-glycerylphosphorylethanolamine (GPE) were found to co-chromatograph with U 3 and U 4 respectively.

Since the acid hydrolysis of GPC and GPE yields glycerophosphates and free base at similar rates (15), this procedure could not be used to confirm the identities of U 3 and U 4. However, in alkali their rates of hydrolysis differ appreciably (15). Mixtures of labelled unknown and corresponding carrier ester were accordingly hydrolyzed in 0.1 *N* sodium hydroxide at 100° C., aliquots were removed at intervals, separated by ionophoresis, and the activties present as GPC and GPE and as glycerophosphate counted directly on the paper. The spots were then cut from the paper and their phosphate contents determined after ashing. The resulting curves are shown in Fig. 4, and confirm the identities of U 3 and U 4.

Attempts to identify on autoradiograms the corresponding serine compounds were unsuccessful. Here, electrical neutrality at pH 6 is impossible because of the carboxyl group of the amino acid residue. None of the unknowns was found to agree with authentic phosphorylserine as regards its properties on paper.

Acid hydrolysis was also used to confirm the identities of glucose-1-phosphate (G1P) and mannose-6-phosphate (M6P). Hydrolysis curves are shown in Fig. 5.

The identity of UDPG was checked by co-chromatography in solvent 3 (see Methods), in which decomposition occurs to glucose-1,2-*cyclic*-phosphate (4). In the other solvents, however, the presence of components other than

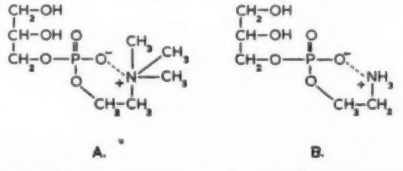
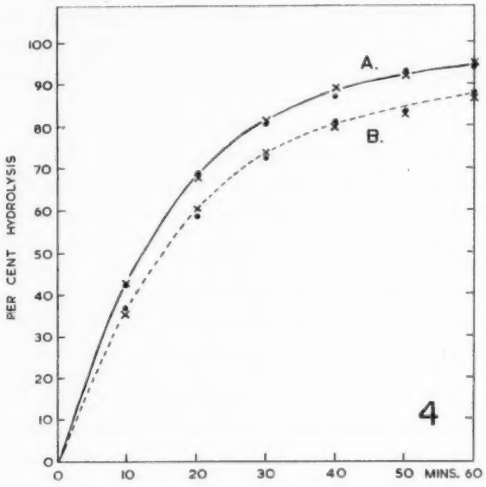


FIG. 3. Zwitterion structures of (A) L-α-glycerylphosphorylcholine, and (B) L-α-glycerylphosphorylethanolamine.



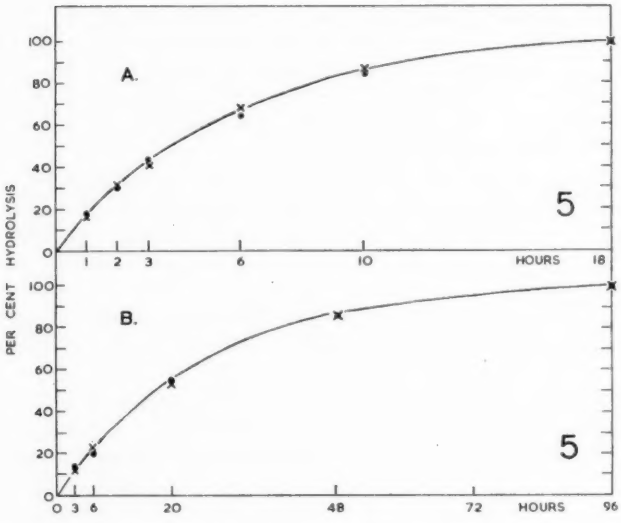


FIG. 4. Hydrolysis curves for (A) L-α-glycerylphosphorylcholine and U 3, and (B) L-α-glycerylphosphorylethanolamine and U 4, in *N* NaOH at 100° C. Values expressed as percentages of total phosphate. Solid circles: chemical determination of glycerophosphate release; crosses: radioactivity measurement of glycerophosphate release.

FIG. 5. Hydrolysis curves for mixtures of authentic and extracted (A) glucose-1-phosphate, and (B) mannose-6-phosphate. Conditions: (A) 0.25 *N* HCl at 37° C.; (B) *N* HCl at 100° C. Symbols as for Fig. 2.

UDPG was demonstrated. Similarly, the spots labelled ADP and ATP were found to contain small amounts of other unidentified phosphates.

U 5 and U 6, when chromatographed in developers 1 and 2, gave spots identical with P_i. When subjected to two-dimensional ionophoresis and chromatography, each yielded spots corresponding to P_i, U 5, and U 6, indicating that they are probably ionic species of orthophosphate.

None of the other unknowns has as yet been identified.

Effects of Various Methods of Killing and Extraction

A previous communication (13) has described the formation of alkyl phosphates in wheat leaf tissue when alcohols are used as killing agents. Thus when methanol is used, a small amount of monomethyl phosphate is found in the extract, while when ethanol is employed this is replaced by monoethyl phosphate (U 7 in Fig. 1). The formation of these alkyl esters has been further studied by testing different methods of killing and extracting, using comparable samples of leaf material.

When boiling alcohol is used, the levels of alkyl phosphate in the extracts are low. However, when leaf material is mascerated in cold (−70° C.) alcohol, and the temperature allowed to rise to ambient, the extracts contain a larger proportion of their total activity as alkyl ester. When intact leaves are immersed in cold alcohol, and the temperature allowed to rise slowly, penetration of the tissue is slow and extraction incomplete, but the yield of alkyl phosphate is considerably higher, as shown in Table I. The greater production of alkyl phosphate in the last case is undoubtedly the result of the slow rate of penetration by the alcohol, with subsequent delay in killing, allowing more esterification to occur before the leaf protein is denatured.

TABLE I

YIELD OF MONOETHYL PHOSPHATE (EtP) ON KILLING INTACT WHEAT LEAF TISSUE WITH ETHANOL. RESULTS OF A TYPICAL EXPERIMENT

	Activity in EtP	
	c.p.m./mg. dry wt.	% total activity
Boiling ethanol	1259	3.3
Cold (−70° C.) ethanol	2921	11.7

In further experiments, leaf pieces were killed by treatment with hot methanol vapor, and then extracted with boiling 80% ethanol. The only alkyl ester present in the extract was the monomethyl ester. Conversely, when ethanol vapor was used to kill the tissue which was then extracted with methanol, only monoethyl phosphate was formed, again suggesting that esterification requires the presence of undenatured leaf protein.

The proposed mechanism for the formation of alkyl phosphates (13) involves ATP according to the equation:

$$\text{Alkyl-OH} + \text{ATP} \rightarrow \text{Alkyl-O-P} + \text{ADP}$$

Attempts to isolate the enzyme catalyzing this reaction have been unsuccessful. However, indirect evidence for the presence of such a system comes from the observation that the levels of extractable ATP in wheat leaves supplied with radiophosphate in both light and dark are lower than those of ADP. Such a situation would arise if, immediately prior to inactivation, some ATP is utilized in alkyl phosphate formation. The levels of ATP, ADP, and ethyl phosphate from a typical experiment are shown in Table II.

TABLE II

LEVELS (10^3 C.P.M./MG. DRY WT.) OF ATP, ADP, AND MONOETHYL PHOSPHATE IN GREEN LEAF TISSUE SUPPLIED WITH P^{32}-ORTHOPHOSPHATE BY VACUUM INFILTRATION

Time from infiltration, minutes	ATP	ADP	EtP
1	0 (0)	1 (8)	2 (20)
5	12 (5.2)	61 (26)	20 (9)
20	48 (4.2)	179 (16)	72 (6.4)
60	122 (4)	430 (16)	76 (2.8)

NOTE: Tissue killed by immersion in boiling ethanol. Figures in parentheses are percentages of total organic phosphate.

To date, the only reported occurrence of alkyl phosphates in plants is in the yeast cell, in which small amounts of ethyl phosphate are formed from ethanol in the culture medium (17). In higher plants, ethanol formation is uncommon and is usually restricted to fruits and storage tissues (various workers cited by James (9)). The production of ethyl phosphate and its subsequent metabolism by reactions as yet unknown could provide a means for its removal from the tissue.

Two other implications of alkyl phosphate formation in higher plants must be mentioned. Firstly, the possibility exists that such phosphates might act as alkyl-group donors in the presence of suitable enzymes. Secondly, their production as artifacts during the killing of tissues with alcohols must be taken into consideration in studies of the dynamics of phosphate metabolism in vivo, such as those undertaken by Goodman *et al.* (5) and Bradley (3). The information presented above shows that under certain conditions considerable esterification may occur, and if this is at the expense of ATP, corrections must be made to account for the diminution of the latter

Acknowledgments

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THE EFFECT OF VARIOUS METHODS OF BREAKING RESISTANCE ON STEM RUST REACTION AND CONTENT OF SOLUBLE CARBOHYDRATE AND NITROGEN IN WHEAT LEAVES[1]

F. R. FORSYTH[2] AND D. J. SAMBORSKI[2]

Abstract

The resistance of Khapli wheat to a number of races of stem rust, *Puccinia graminis* (Pers.) f. sp. *tritici* (Erikss. and Henn.), was broken after treatment with maleic hydrazide, DDT, detaching the leaves, and "searing" (heat treatment at the base of the leaf). In addition, the reaction of several other varieties to a number of races of stem rust was altered from resistant to susceptible by detaching and searing.

DDT and searing caused marked increases in carbohydrate and soluble nitrogen levels, while maleic hydrazide caused smaller increases. There were no differences in carbohydrate levels, and only a slight difference in soluble nitrogen, between plants kept at 65° and 75° F., the temperatures at which McMurachy is respectively resistant and susceptible to rust. Insoluble nitrogen decreased after searing and DDT at rates approximately equal to those of the control. Insoluble nitrogen decreased at a faster rate in leaves kept at 75° F. than at 65° F. It remained fairly constant in leaves treated with maleic hydrazide.

The data support the concept that substrate availability is of major importance in rust development.

Introduction

The rust reaction of wheat varieties is sufficiently stable under a wide range of environmental conditions to permit the identification of rust races on selected hosts. Nevertheless it is well known that environmental factors, particularly temperature, may influence the reaction of wheat plants to rust (2, 6, 8). Most studies on the effects of temperature have been carried out with considerable differences in temperature, but in certain cases, a rise of only a few degrees will change the host reaction from resistant to susceptible (4). Other factors, such as the levels of nitrogen and potassium supplied to plants (7) and the physiological age of infected plant parts (14), also influence rust reaction.

Khapli (*Triticum dicoccum* Schubler), a highly resistant variety which has been used extensively in studies of host–parasite relations, has been made susceptible by a variety of methods. These include treatments with DDT (dichlorodiphenyltrichloroethane) (1, 3, 12), maleic hydrazide (17), ionizing radiations (18), excesses of Zn^{++}, Co^{++}, and Mn^{++} (5), and detaching of the leaves (16). The metabolic disturbances which occurred in treated plants resulted in considerably increased levels of carbohydrate and amino acids (3, 16, 17).

The purpose of this study was to determine whether or not there is a common factor in the metabolic changes brought about by the various methods of altering host resistance to stem rust of wheat.

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Methods

First leaves of 7- to 8-day-old wheat seedlings were used for all studies. A number of races and subraces of stem rust,* *Puccinia graminis* (Pers.) f. sp. *tritici* Erikss. and Henn., were used.

Resistance of wheat leaves was altered by detaching leaves and floating them on water, 40 p.p.m. benzimidazole, and benzimidazole with 2% glucose, treating the soil or sand with 100 p.p.m. maleic hydrazide daily commencing 6 days before inoculation, spraying the leaves with 640 p.p.m. of DDT, "searing", and changing the temperature. Searing of the leaves consisted of damaging the bases of the first leaves with a hot spatula. The seared area became necrotic but the leaf remained turgid and green for more than a week, indicating that the xylem was intact.

McMurachy (*T. vulgare* (Vill.) Host) seedlings, 7 to 8 days after planting, were placed in two growth chambers with controlled environment at 65° F. and 75° F. The seedlings were resistant to race 15B of stem rust at 65° F. and susceptible at 75° F.

Alcohol-soluble carbohydrates were determined as described in (16). Nitrogen determinations were carried out, by the procedures given in (16), on 150 leaf disks cut with a 3-mm. stainless steel cutter. The water-soluble fraction of the alcohol extract was used for determination of "soluble nitrogen". Insoluble nitrogen was determined on the alcohol-extracted disks.

Results

Comparison of Rust Reaction on Normal and Treated Leaves

Khapli wheat is normally resistant to 228 of 232 races of stem rust (21). It was shown at Winnipeg that seedlings of this variety when treated with 100 p.p.m. maleic hydrazide were susceptible to 18 of 19 races used. Khapli seedlings, sprayed with 640 p.p.m. DDT, became susceptible to the 17 races that were used. In addition, both detaching of the leaves and searing break the resistance of the treated Khapli leaves to most races that have been used. It is probable that with appropriate treatment Khapli can be made susceptible to most of the stem rust races.

Several workers have concluded (see (10)) that both the cells of the host at the infection site and the mycelium of the rust fungus are dead when the resistant reaction is expressed. We have shown that treatment of Khapli with DDT results in increased rust development when applied as long as 11 days after inoculation. Thus the mycelium of the fungus was still capable of growth after the resistant reaction had been established.

Khapli leaves which are normally resistant to race 15B of stem rust became susceptible when they were detached and floated on water or 40 p.p.m. benzimidazole containing glucose. The detached leaves were resistant when floated on 40 p.p.m. benzimidazole (16). Table I shows that this was also true for

*The races and subraces used were: 1, 2, 11-1, 15B1, 15B1L, 15B4, 29-2, 34, 48, 56, and 59C as described in (9) and (21).

TABLE I

COMPARISON AT 75° F. OF NORMAL RUST REACTIONS WITH REACTIONS ON DETACHED LEAVES FLOATED ON 40 P.P.M. BENZIMIDAZOLE AND ON 40 P.P.M. BENZIMIDAZOLE CONTAINING 2% GLUCOSE

Wheat variety	Rust reactions																							
	Race 56			Race 34			Race 29-2			Race 15B1L			Race 15B1			Race 11-1			Race 59C			Race 15B4		
	N	B	BG	N	B	BG	N	B	BG	N	B	BG	N	B	BG	N	B	BG	N	B	BG	N	B	BG
McMurachy	S	S	S	S	S	S	—	—	—	S	S	S	S	S	S	S	S	S	MS	S	S	S	S	S
Bowie	R	R	S	MS	X	S	—	—	—	R	S	S	R	R	R	R	R	S	—	—	—	R	R	S
Kenya Farmer	R	—	R	MS	R	S	R	R	X	R	R	S	R	R	S	R	R	R	R	R	R	R	R	R
Mindum	R	R	S	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	R	R	S	—	—	—
Einkorn	R	R	S	X	X	S	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Vernal	R	R	S	X	R	S	R	R	S	—	—	—	—	—	—	R	R	MS	R	R	R	—	—	—
Khapli	R	R	S	R	R	S	R	R	S	R	R	S	R	R	S	R	R	S	R	R	S	R	R	S
Lee	R	X	S	S	R	S	R	R	S	—	—	—	—	—	—	R	R	MS	R	—	MS	—	—	—
Selkirk	R	X	S	—	—	—	—	—	—	—	—	—	—	—	—	R	R	S	R	R	MS	—	—	—

NOTE: N—normal, B—benzimidazole, BG—benzimidazole and 2% glucose, S—susceptible, R—resistant, MS—moderately susceptible, X—mesothetic reaction.

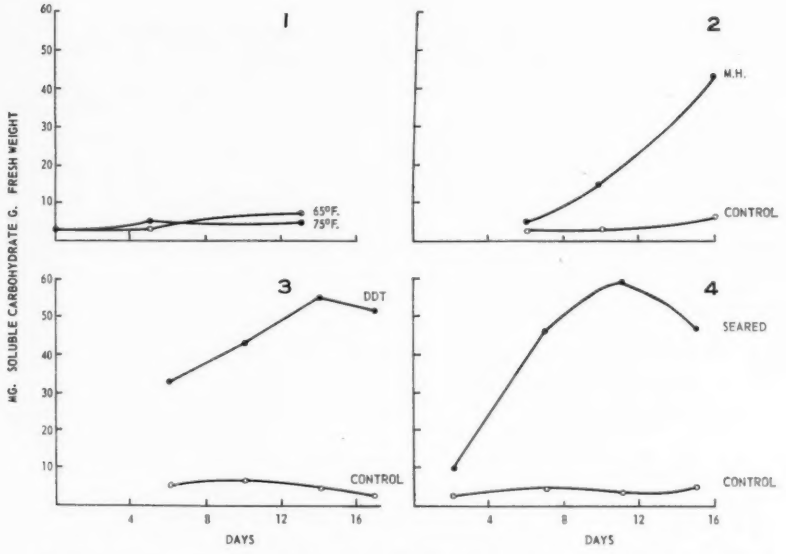
TABLE II

COMPARISON OF NORMAL RUST REACTIONS WITH REACTIONS ON SEARED LEAVES

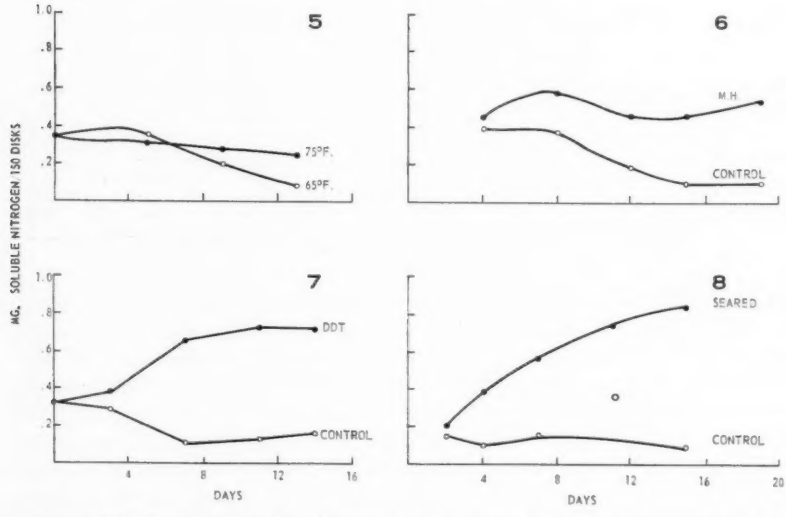
Wheat variety	Rust reactions											
	Race 56		Race 59C		Race 1		Race 2		Race 15B1L		Race 48	
	N	T	N	T	N	T	N	T	N	T	N	T
Marquis	S	S	—	—	MS	S	MS	S	S	S	MS	S
Reliance	S	S	—	—	O	O	O	O	S	S	O	O
Arnautka	S	S	—	—	MS	S	R	S	S	S	R	MS
Mindum	R	MS	—	—	R	S	R	MS	S	S	R	S
Einkorn	R	MS	—	—	R	S	R	MS	R	S	R	S
Vernal	R	MS	—	—	R	S	R	MS	S	S	R	S
Lee	S	S	S	S	S	S	—	—	—	—	R	MS
Golden Ball	X	S	R	S	R	S	—	—	—	—	R	MS
Selkirk	R	S	S	S	MS	S	—	—	—	—	S	S
Kenya Farmer	R	MS	R	S	R	S	—	—	—	—	R	S
Bowie	R	S	R	S	R	S	—	—	—	—	R	MS
McMurachy	R	MS	R	MS	R	S	—	—	—	—	R	MS

NOTE: N—normal, T—seared, O—immune.

other host–race combinations. Normally resistant reactions remained resistant on 40 p.p.m. benzimidazole but in most cases they were susceptible on benzimidazole with 2% glucose. Probably the few reactions which were resistant on 40 p.p.m. benzimidazole plus 2% glucose could be made susceptible by modifying the method. In fact, an example in support of this conclusion is shown in Table II. Leaves of Kenya Farmer (*T. vulgare*) inoculated with race 59C were resistant when detached and floated on benzimidazole plus glucose but became susceptible after searing. Tables I and II show that the reaction of several varieties of wheat to a number of races of stem rust can be altered from resistant to susceptible by appropriate treatment. A notable exception has been the zero reaction on Reliance (*T. vulgare*).



FIGS. 1–4. Levels of total soluble carbohydrates in wheat leaves following treatments which induce susceptibility to stem rust. Fig. 1, temperature; Fig. 2, maleic hydrazide; Fig. 3, DDT; Fig. 4, seared.



FIGS. 5–8. Levels of soluble nitrogen in wheat leaves following treatments which induce susceptibility to stem rust. Fig. 5, temperature; Fig. 6, maleic hydrazide; Fig. 7, DDT; Fig. 8, seared.

Comparative Analyses of Normal and Treated Wheat Leaves

Changes in alcohol-soluble carbohydrate and soluble and insoluble nitrogen were determined after treatments which break resistance. McMurachy wheat was used for the temperature studies and Khapli in all other experiments.

The curves in Figs. 1 to 4 show the levels of soluble carbohydrate and those in Figs. 5 to 8 show the levels of soluble nitrogen in the normal and treated leaves. Soluble carbohydrates increased rapidly after DDT and searing and less rapidly after maleic hydrazide treatments. The temperatures used had little effect on carbohydrate levels. The rates and extent of change of soluble nitrogen were similar to those for carbohydrates. Rapid and marked increases occurred after DDT and searing whereas after maleic hydrazide the increases were less rapid and less marked. McMurachy plants kept at 75° F. contained more soluble nitrogen than those at 65° F. The rate of increase and extent of these changes are interesting since DDT and searing break the resistance of Khapli when applied after inoculation, while maleic hydrazide usually does not. Maleic hydrazide is just as effective as the other methods if treatments are started several days before inoculation.

TABLE III

LEVELS OF INSOLUBLE NITROGEN IN WHEAT LEAVES FOLLOWING TREATMENTS WHICH INDUCE SUSCEPTIBILITY TO STEM RUST (MG. NITROGEN PER 150 LEAF DISKS)

Seared*			DDT*			Maleic hydrazide*			Temperature, °F.*		
Days	Control	Treated	Days	Control	Treated	Days	Control	Treated	Days	65*	75*
2	0.93	0.95	0	0.95	0.93	4	1.11	0.98	0	1.04	1.04
4	0.81	0.79	3	0.79	0.83	8	0.83	0.96	5	0.88	0.63
7	0.71	0.59	7	0.59	0.73	12	0.76	1.06	9	0.79	0.55
11	0.55	0.52	11	0.52	0.58	15	0.62	0.96	13	0.63	0.41
15	0.41	0.47	15	0.47	0.47	19	0.64	1.02			

*Khapli was used in the seared, DDT, and maleic hydrazide treatments and McMurachy in the temperature treatments.

The results in Table III show how the treatments affect levels of insoluble nitrogen. Insoluble nitrogen decreased after searing and DDT treatments at rates approximately equal to those of the controls. It decreased more rapidly at 75° F. than at 65° F. It remained about constant in the maleic hydrazide treated leaves. In every treatment where it occurs, the reduction in amount of insoluble nitrogen can be accounted for by the appearance of more soluble nitrogen in the tissues. The maleic hydrazide treatment not only maintains a high level of insoluble nitrogen but also maintains a high level of soluble nitrogen.

Discussion

The resistance of many host varieties to certain races was broken after detaching of the leaves and searing, treatments which interfere with transport away from affected leaves and result in marked increases in substrate. Maleic hydrazide treatment was applied to only a few host–race combinations but the resistance of most of these was broken. Only Khapli and a few other

varieties, all of which have 14 chromosomes, are affected by DDT (1); Khapli becomes susceptible to many rust races. Present evidence indicates that DDT interferes with transport, resulting in the accumulation of substrate in the treated leaves. While superficially the treatments which break resistance appear quite different, analytical studies indicate that a similar effect, namely a marked increase in carbohydrate and soluble nitrogen, occurs in each case. Apparently, any treatment which results in massive accumulation of substrate in the wheat plant will tend to induce susceptibility to stem rust.

Substantial changes in resistance at different temperatures are shown by some varieties (8). Although in the present study there was little effect of temperature on substrate, it is possible that certain critical constituents accumulate without any marked change in a fraction such as soluble nitrogen.

The change in resistance as a result of treatment may result from the removal of inhibiting substances or the increase in availability of essential substrate. Phenols have been implicated in host–parasite relations because of their ubiquity and well-known toxicity to microorganisms. Apart from their toxic characteristics phenols may, through their action in the indoleacetic acid oxidase system (13), alter indoleacetic acid levels and thereby affect host–parasite relations (19).

The level of phenols may in turn be altered by glucoside metabolism (11). The increase in level of soluble carbohydrates resulting from most treatments which induce susceptibility would be expected to decrease the level of free phenols through the formation of glucosides. However it is not possible to evaluate critically the role of phenols in rust development at present.

It has been suggested that infected cells compete for substrate with other sites of high metabolic activity and that successful parasitism by rust fungi requires the early establishment of a local field of dominance (17, 19, 20). This field of dominance, corresponding to a metabolic "sink" (17, 19, 22), is obtained on susceptible but not on resistant varieties (19). There is no direct evidence on specific nutritional requirements of rust fungi but it has recently been inferred that availability of amino acids is of particular importance to rust development (16). Some support for this view can be derived from the work of Gassner and his associates. Wheat plants grown with high nitrogen or low potassium supply were more susceptible to leaf rust than those grown with low nitrogen or high potassium (7). Richards and Berner (15) showed that low potassium supply resulted in the accumulation of amino acids in barley leaves; presumably a similar result would be found with wheat.

The results of the present study support the concept that substrate availability is of major importance in rust development but do not indicate whether any individual constituent of the substrate is of particular significance.

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FURTHER EVIDENCE FOR THE VALIDITY OF THE DEGRADATION OF C^{14}-LABELLED ASPARTIC ACID ISOLATED FROM DETACHED LEAVES[1]

C. D. NELSON

Nelson and Krotkov (3) have reported on the position labelling of aspartic acid isolated from excised leaves of *Vicia faba* which had fixed C^{14} during photosynthesis. They found that C_2 plus C_3 was more radioactive than C_1 plus C_4. No known biosynthetic pathway can explain this unexpected labelling. The original paper reported that 2 moles of CO_2 were produced for each mole of aspartic acid reacted with ninhydrin. However, there was still the possibility that the method did not remove the β-carboxyl quantitatively and that one of the moles of CO_2 produced in the reaction originated totally or partly from C_2 plus C_3. To test the validity of the method, aspartic acid specifically labelled in position 4 (Tracerlab, Waltham, Mass.) was degraded using the methods described (3).

The radioactive aspartic acid was mixed with carrier in aqueous solution. One aliquot was subjected to total combustion by the method of Baker *et al.* (1) and a second aliquot was decarboxylated using ninhydrin (3). The amount of CO_2 produced in each reaction was determined by titration and counted as $BaCO_3$. Samples were counted to a standard error of 3% using a gas flow counter with micromil window (model D47, Nuclear, Chicago). Counting data were corrected for self-absorption by the method of Karnovsky *et al.* (2).

TABLE I

NINHYDRIN DEGRADATION OF ASPARTIC ACID-4-C^{14}

	Weight of aspartic acid, mg.	Calc. carbon, mg.	Found carbon, mg.	Recovery, %	Specific activity of recovered C, c.p.m. per mg. C	Specific activity of C_4, c.p.m. per mg. C
Total combustion, $C_1 + C_2 + C_3 + C_4$	15.46	5.56	5.52	99.4	4,020	16,080
Ninhydrin decarboxylation, $C_1 + C_4$	30.92	5.56	5.46	98.2	8,165	16,330

NOTE: Total combustion and ninhydrin decarboxylation were carried out in duplicate. Counting results are the averages of three planchets.

From the table it is evident that in the ninhydrin reaction 2 moles of CO_2 were produced from each mole of aspartic acid. In addition, all of the C^{14} from C_4 was recovered in the CO_2 produced. The specific activity data show that there was no contamination of carbon from C_1 plus C_4 by carbon from the rest of the molecule.

It is concluded that the methods as originally outlined gave a true picture of the labelling in aspartic acid.

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